

THE PATHWAY TAKEN BY 5S RNA IN OOCYTES OF
XENOPUS LAEVIS

A thesis
submitted in fulfilment of
the requirements for the degree
of
Doctor of Philosophy
in
Cellular and Molecular Biology
at the
University of Canterbury

MELANIE NORTH

1996

THESIS

PL

668

. E265

. N866

To my grandparents,
Martha and Lewen Hanna, and
Joan and Victor North.

TABLE OF CONTENTS

ABSTRACT	1
CHAPTER 1	
GENERAL INTRODUCTION	2
1.1 THE RIBOSOMAL RNAS	3
1.2 OOGENESIS IN <i>XENOPUS LAEVIS</i>	5
1.3 PATHWAY TAKEN BY 5S RNA IN <i>XENOPUS</i> OOCYTES ...	6
1.4 THE PRESENT STUDY	9
CHAPTER 2	
MATERIALS AND METHODS	10
2.1 MATERIALS	10
I. Plasmids	10
II. Antisera	12
2.2 <i>IN VITRO</i> TRANSCRIPTION OF RNA	12
I. ³² P-labelled 5S RNAs	12
i. Method A	12
ii. Method B	13
II. ³² P-labelled U1 snRNA and U3 snoRNA	14
III. ³³ P-labelled RNAs	14
IV. DIG-labelled Antisense RNA Probes	14
2.3 <i>IN VITRO</i> SYNTHESIS OF ³⁵ S-LABELLED L5	15
2.4 OOCYTE ISOLATION AND PREPARATION	15
2.5 MICROINJECTION	16
I. Equipment	16
II. Procedure	16
2.6 IMMUNOPRECIPITATION ASSAYS	17
I. Whole Oocytes	17
II. Nuclear Fractions	18

2.7	NON-DENATURING GEL ELECTROPHORESIS ASSAYS	19
2.8	NUCLEOLAR LOCALISATION ASSAYS	20
	I. Biochemical Fractionation	20
	i. RNA extraction and analysis	20
	ii. Protein extraction and analysis	21
	II. <i>In Situ</i> Localisation	21
	i. Preparation of sections	21
	ii. Autoradiography and staining	22
2.9	WESTERN ANALYSIS	23
	I. Preparation of Samples	23
	II. Electrophoresis and Transfer	24
	III. Immunodetection	24
2.10	NORTHERN ANALYSIS	25
	I. Preparation of Samples	25
	II. Electrophoresis and Transfer	25
	III. Hybridisation	26
	i. Antisense RNA probes	26
	ii. DNA probes	26
	IV. DIG Chemiluminescent Detection	27
2.11	CYTOSKELETON PREPARATION	27
	I. General Procedure	28
	i. Total RNA extraction	28
	ii. Immunoprecipitation	28
	II. Enrichment of Microtubules	29
	III. Enrichment of Intermediate Filaments	29
	IV. Enrichment of Microfilaments	29
2.12	ISOLATION OF INTRACELLULAR MEMBRANES	30
	I. Total RNA Extraction	30
	II. Immunoprecipitation	30

CHAPTER 3

SEQUENCE AND STRUCTURAL REQUIREMENTS OF 5S RNA FOR PROTEIN ASSOCIATIONS AND RIBOSOME ASSEMBLY

IN <i>XENOPUS</i> OOCYTES	32
3.1 INTRODUCTION	32
I. Secondary and Tertiary Structure of 5S RNA	33
II. TFIIIA Binding	36
III. L5 Binding	36
IV. 5S RNA within the Ribosome: Structure and Function ...	38
V. Description of 5S RNA Mutants used in this Study	40
3.2 RESULTS	43
I. Characterisation of Antisera	43
II. Analysis of Mutant RNAS for Incorporation into 7S RNPS and 60S Ribosomal Subunits by Immunoprecipitation	44
i. 7S RNP formation	44
ii. 60S ribosomal subunit incorporation	46
III. Analysis of Mutant RNAS for 7S RNP and 5S RNP Formation by Non-denaturing PAGE	48
i. 7S RNP formation	48
ii. 5S RNP formation	49
3.3 DISCUSSION	53
I. TFIIIA Binding	53
II. L5 Binding	54
III. Assembly into 60S Ribosomal Subunits	55
i. Mutant 10-13	55
ii. Mutants Δ 49,50 and Δ 63	56
iii. Mutant 96-101	58
iv. Functional implications	59

CHAPTER 4

DISTRIBUTION OF 5S RNA WITHIN THE *XENOPUS* OOCYTE

NUCLEUS	61
4.1 INTRODUCTION	61
I. Structure and Function of the Nucleolus	62
II. Nucleolar Proteins	66
III. Nucleolar Targeting	70
i. Nuclear import	70
ii. Nucleolar localisation	72
a. Proteins	72
b. RNA	75
IV. The Present Investigation	76
4.2 RESULTS	78
I. Nucleolar Localisation of Endogenous 5S RNA	78
II. Nucleolar Localisation of Exogenous 5S RNA	79
III. Nucleolar Localisation 5S RNA Mutants	83
IV. Nucleolar Localisation of Ribosomal Protein L5	89
V. 5S RNA-protein Associations in the Nucleus	92
4.3 DISCUSSION	100
I. Requirements for the Nucleolar Localisation of 5S RNA ..	100
II. Nucleoplasmic 5S RNA	103
III. Nucleolar Localisation of Mutants Defective for Ribosome Assembly	104
IV. Pathway of 5S RNA to the Ribosome	107

CHAPTER 5

7S RNP LOCALISATION IN THE CYTOPLASM	110
5.1 INTRODUCTION	110
I. Evidence for the Role of the Cytoskeleton in Localising Proteins and RNA	112
i. Protein retention	112
ii. RNA association with the cytoskeleton	113
a. Somatic cells	113
b. <i>Drosophila</i> oocytes	115
c. <i>Xenopus</i> oocytes	116
d. Requirements for localisation	117
iii. RNP associations with the cytoskeleton	118
II. Evidence for the Role of Intracellular Membranes in Localising Proteins and RNA	120
i. Protein retention	120
ii. RNA association with intracellular membranes	121
III. Description of the Cytoskeleton and Membrane Systems in <i>Xenopus</i> Oocytes	122
i. The cytoskeleton	123
ii. The internal membrane system	124
5.2 RESULTS	126
I. 7S RNPs are not Associated with the Oocyte Cytoskeleton	126
II. 7S RNPs are not Associated with Oocyte Intracellular Membranes	131
5.3 DISCUSSION	133
I. Lack of Nuclear Retention Sites for 7S RNPs	134
II. 7S RNP Dimerisation	135
III. Binding of an Inhibitory Subunit to 7S RNPs	136
IV. Post-translational Modification	137
V. Cytoplasmic Retention of other Molecules in <i>Xenopus</i>	139

CHAPTER 6

GENERAL DISCUSSION	141
6.1 REQUIREMENTS FOR THE PATHWAY TAKEN BY 5S RNA DURING OOGENESIS	141
6.2 SUBCELLULAR LOCALISATION OF 5S RNA	144
6.3 BEHAVIOUR OF SOMATIC-TYPE 5S RNA IN OOCYTES ...	145
6.4 THE PATHWAY TAKEN BY 5S RNA	146
6.5 5S RNA/L5-CONTAINING COMPLEXES	147
ACKNOWLEDGEMENTS	150
REFERENCES	151
APPENDICES	187
I SUMMARY OF MUTANT 5S RNA PHENOTYPES	188
II NUCLEOLAR LOCALISATION OF 5S RNA MUTANTS	189
III JOURNAL PUBLICATIONS	190

LIST OF FIGURES

Figure 1-1	Schematic representation of the components of the eukaryotic ribosome.	3
Figure 1-2	Pathway taken by 5S RNA in <i>Xenopus</i> oocytes	7
Figure 3-1	Secondary and tertiary structure of <i>Xenopus laevis</i> oocyte-type 5S RNA	35
Figure 3-2	Diagram of prokaryotic 50S large ribosomal subunit, showing proposed location of 5S RNA and binding proteins	40
Figure 3-3	Secondary structure of <i>Xenopus laevis</i> oocyte-type 5S RNA showing mutant nucleotide substitutions and deletions.	42
Figure 3-4	Immunoprecipitation of endogenous oocyte RNAs with anti-TFIIA and anti-60S subunit antisera	45
Figure 3-5	Assembly of mutant 5S RNAs into 7S RNPs and 60S ribosomal subunits assayed by immunoprecipitation	47
Figure 3-6	Non-denaturing gel electrophoresis of mutant 5S RNAs showing formation of 5S RNPs and 7S RNPs	50
Figure 4-1	Ribosome biosynthesis in a typical eukaryotic cell	65
Figure 4-2	Secondary structure of <i>Xenopus laevis</i> 5S RNA showing somatic-specific substitutions.	77

Figure 4-3 Distribution of the nucleolar protein B23/NO38 and of rRNA within the <i>Xenopus</i> oocyte nucleus	80
Figure 4-4 Nucleolar localisation of endogenous 5S RNA throughout oogenesis in <i>Xenopus</i> oocytes	80
Figure 4-5 Nucleolar localisation of exogenous RNAs	82
Figure 4-6 <i>In situ</i> nucleolar localisation of exogenous RNAs in <i>Xenopus</i> oocytes	84
Figure 4-7 Nucleolar localisation of mutant 5S RNA molecules	86
Figure 4-8 Summary of nucleolar localisation of 5S RNA mutants	87
Figure 4-9 <i>In situ</i> nucleolar localisation of 5S RNA mutants	88
Figure 4-10 Nucleolar localisation of ribosomal protein L5	90
Figure 4-11 <i>In situ</i> nucleolar localisation of ribosomal protein L5	91
Figure 4-12 Non-denaturing gel electrophoresis of oocyte fractions	93
Figure 4-13 Characterisation of <i>Artemia</i> anti-L5 antiserum	95
Figure 4-14 Immunoprecipitation of oocyte nuclear fractions	97
Figure 4-15 Immunoprecipitation of oocyte nucleoplasmic fractions	99
Figure 4-16 Model for the pathway taken by oocyte-type and somatic-type 5S RNAs in <i>Xenopus</i> oocyte nuclei	109

Figure 5-1	5S RNA is not contained within the cytoskeletal fraction of oocytes	128
Figure 5-2	7S RNPs are not contained within the cytoskeletal fraction of oocytes	130
Figure 5-3	Small amounts of 5S RNA are contained in the cytoskeletal fraction after extraction with a low salt extraction buffer	130
Figure 5-4	7S RNPs are not contained within the cytoskeletal fraction after extraction with a low salt buffer	130
Figure 5-5	Small amounts of 5S RNA are associated with oocyte intracellular membrane fractions	132
Figure 5-6	7S RNPs are not associated with oocyte intracellular membrane fractions	132
Figure 5-7	Possible mechanisms for the cytoplasmic localisation of 7S RNPs ..	138

LIST OF TABLES

Table 3-1	Summary of incorporation of mutant 5S RNAs into oocyte RNPs	52
Table 4-1	Nucleolar proteins involved in ribosome assembly	68
Table 4-2	Nucleolar localisation of 5S RNA throughout oogenesis	81
Table 6-1	5S RNA and/or L5-containing complexes	148

LIST OF ABBREVIATIONS

5S RNA	5S ribosomal ribonucleic acid
5S RNP	5S ribosomal RNA bound to ribosomal protein L5
7S RNP	5S ribosomal RNA bound to transcription factor IIIA
BSA	bovine serum albumin
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
EDTA	ethylene dinitrilo tetraacetic acid
EF-1 α	elongation factor 1 α
ETS	external transcribed spacer
HIV	human immunodeficiency virus
hnRNA	heterogeneous ribonucleic acid
hsp90	heat shock protein 90
HTLV	human T-cell leukaemia virus
ITS	internal transcribed spacer
kD	kilodalton
mRNA/mRNP	messenger ribonucleic acid/ribonucleoprotein particle
NLS	nuclear localisation sequence
NOR	nucleolus organiser region
PAGE	polyacrylamide gel electrophoresis
rDNA	ribosomal deoxyribonucleic acid
Rh(phen) ₂ phi ³⁺	bis(phen-anthroline)(phenanthrenequinone diimine) rhodium III
RNP	ribonucleoprotein particle
RRE	Rev-responsive element
rRNA	ribosomal ribonucleic acid
scRNA	small cytoplasmic ribonucleic acid
SDS	sodium dodecyl sulphate
snRNA	small nuclear ribonucleic acid
snoRNA	small nucleolar ribonucleic acid
SV-40	Simian virus 40

TAR	<i>trans</i> -activation-responsive region
TFIIIA	transcription factor IIIA
tRNA	transfer ribonucleic acid
UBF	upstream binding factor
UTR	untranslated region

ABSTRACT

In *Xenopus* oocytes, 5S ribosomal RNA (5S RNA) is synthesised prior to other ribosomal components, and stored in the cytoplasm in ribonucleoprotein particles (RNPs). At vitellogenesis, when ribosome assembly begins, 5S RNA is imported into the nucleus and targeted to the amplified nucleoli for assembly into the 60S ribosomal subunit. In this thesis, I have investigated some of the steps of this pathway taken by 5S RNA.

In vivo assays using a series of mutant 5S RNAs revealed that only one mutant, with base substitutions in loop A, was defective for binding the 7S RNP storage protein, TFIIA. All mutants were capable of binding to ribosomal protein L5, a precursor to ribosome assembly. Four of the mutants tested were defective for incorporation into 60S subunits, possibly due to a loss of recognition sites for interactions with other ribosomal proteins. Nucleolar localisation studies showed that the defective ribosome incorporation of these mutants was not due to defective nucleolar targeting. Taken together, these results reveal that different structural features of 5S RNA and different oocyte factors are required for different steps in the pathway taken by 5S RNA.

Nucleolar localisation studies also revealed that a large proportion of oocyte-type 5S RNA and L5 in the nucleus are not associated with nucleoli. In contrast, somatic-type 5S RNA was predominantly associated with nucleoli, suggesting that nuclear factors directly recognise the sequence differences between the two types of 5S RNA. These could be nucleolar components, which have a higher affinity for somatic-type, or nucleoplasmic factors which have a higher affinity for oocyte-type.

Finally, the mechanism by which 7S RNPs are sequestered in the cytoplasm of previtellogenic oocytes was investigated. The results show that neither cytoskeletal or membrane structures are responsible for cytoplasmic retention. Other possibilities for cytoplasmic retention are discussed.

CHAPTER 1

GENERAL INTRODUCTION

The term ribosome was first coined in 1958, to describe 20 to 100S ribonucleoprotein particles (RNPs), by R. H. Roberts, who wrote: "this seems a very satisfactory name, and it has a pleasant sound" (as quoted in Nomura, 1990). Subsequently, ribosomes were shown to be directly responsible for protein synthesis in all animal, plant and bacterial cells. Functional ribosomes are made up of two subunits, in prokaryotes the 30S and 50S subunits, and in eukaryotes the 40S and 60S subunits, which each have different protein and RNA components. As shown in Figure 1-1, the 60S subunit consists of three ribosomal RNAs (rRNAs): the 28S rRNA, 5.8S rRNA and 5S rRNA, as well as approximately 49 ribosomal proteins. The 40S subunit contains the 18S rRNA and approximately 33 ribosomal proteins (reviewed in Hadjiolov, 1985).

The two ribosomal subunits are made and present in the cell in equimolar amounts. Thus, the biogenesis of ribosomes requires the co-ordinate synthesis of four rRNAs and 80 proteins. A fundamental challenge in cellular and molecular biology is understanding how the co-ordinate expression and subsequent assembly of so many molecules is achieved. One approach to studying such a complex structure as the ribosome is to investigate the synthesis and structure of individual ribosomal components. One such component is 5S rRNA and specific RNPs which it forms. The focus of this study is the pathway taken by 5S rRNA, from synthesis to ribosome incorporation, in oocytes of the African Clawed Frog, *Xenopus laevis*.

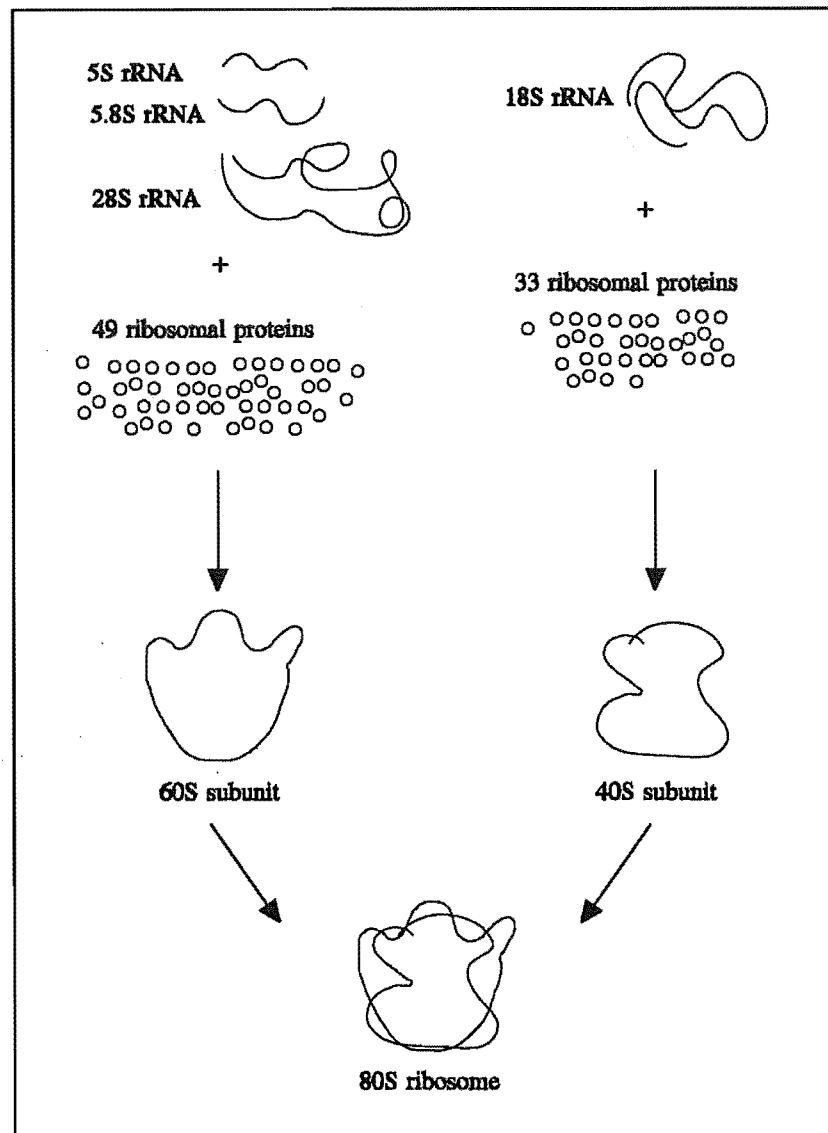


Figure 1-1 Schematic representation of the components of the eukaryotic ribosome.

1.1 THE RIBOSOMAL RNAS

The equimolar synthesis of the 18S, 28S and 5.8S rRNAs is ensured by their placement within a single transcription unit, which is transcribed as a single pre-rRNA molecule by RNA polymerase I. The rRNA genes are highly repeated, being present in 10^2 to 10^3 copies per haploid genome, and are usually grouped in one or several clusters located at specific sites on one or a few chromosomes (reviewed in Hadjiolov, 1985). They are usually looped off the main chromosomal

fibre masses as highly extended threads. These ribosomal DNA (rDNA) loops coalesce with specific proteins to form nucleoli. The rDNA repeat sequences are therefore known as nucleolus organiser regions (NORs). The nucleolus is a non-membrane bound, intranuclear organelle, which is the site of rRNA transcription and processing, and ribosomal subunit assembly (reviewed in Hadjiolov, 1985).

The fourth rRNA, 5S rRNA (referred to hereafter as 5S RNA), is transcribed from another set of tandemly arranged genes which are under the control of RNA polymerase III, and are found on chromosomes that are not nucleolar associated (Hadjiolov, 1985). Initiation of 5S RNA transcription depends on the 5S RNA gene-specific transcription factor IIIA (TFIIIA), which binds tightly to nucleotides 50-84 of the 5S RNA gene (Engelke *et al.*, 1980), as well as two general transcription factors, TFIIB and TFIIC (reviewed in Wolffe and Brown, 1988). 5S RNA is a small molecule of 120 nucleotides and is conserved from bacteria to higher eukaryotes (reviewed in Garrett *et al.*, 1981; Specht *et al.*, 1990).

After transcription in somatic cells, 5S RNA transiently associates with the La protein (Steitz *et al.*, 1988), which is thought to be involved in the termination of transcription of polymerase III transcripts (Gottlieb and Steitz, 1989). 5S RNA is then targeted to the nucleolus and incorporated into the 60S ribosomal subunit. Although it is an essential component of the ribosome, a specific function for 5S RNA has yet to be elucidated (reviewed in Garrett *et al.*, 1981).

When mammalian ribosomes or 60S subunits are treated with EDTA (ethylene dinitrilo tetraacetic acid), a complex of 5S RNA bound to ribosomal protein L5 is released (Blobel, 1971). A similar complex is also released from yeast ribosomes, where the bound protein is called L1 or YL3 (Nazar, 1979). In *Escherichia coli*, the complex released by similar treatment contains 5S RNA and three ribosomal proteins, L5, L18 and L25 (Yu and Wittmann, 1973). Subsequently, these 5S RNA complexes were shown to be precursors to ribosome assembly in HeLa cells (Steitz *et al.*, 1988), *Xenopus* oocytes (Allison *et al.*, 1991) and embryos (Wormington, 1989), yeast (Deshmukh *et al.*, 1993) and *E. coli* (Yu and Wittmann, 1973). In the following text, this group of ribosomal proteins will be referred to as the 5S RNA-binding proteins, as distinguishable from other ribosomal proteins which may interact with 5S RNA in the ribosome, but do not remain associated with 5S RNA after dissociation with EDTA, and do not form precursor RNPs with 5S RNA.

1.2 OOGENESIS IN *XENOPUS LAEVIS*

After fertilisation, the *Xenopus* embryo undergoes twelve very rapid and almost synchronous cleavage divisions, characterised by short interphase periods during which DNA synthesis occurs at a very accelerated rate. During this time there is no detectable RNA transcription, so the egg relies solely on factors which have been stockpiled during oogenesis. These include approximately 10^{12} ribosomes, yolk, maternal messenger RNAs (mRNAs) that are translated in early development, and many proteins (reviewed in Hausen and Riebesell, 1991). The oocyte, therefore, is a highly productive cell, with many specialised mechanisms to allow the synthesis of huge amounts of maternal factors.

Oogenesis in *Xenopus* is a continuous, asynchronous process, and oocytes in all stages of development are present in the ovary at all times during adult life. These oocytes are arrested in the first meiotic prophase, until hormonal stimulation results in the resumption of meiosis and the development of the mature egg (reviewed in Bement and Capco, 1990). Oogenesis has been divided up into six stages based on the anatomy of the developing oocyte (Dumont, 1972). The massive synthesis of ribosomes, together with the large size of the fully grown oocyte (greater than one millimetre) and the accessibility of the oocytes renders them an excellent model system for studying ribosome biogenesis.

Ribosomes are synthesised at a rate 1000 times faster in oocytes than in somatic cells (reviewed in Wormington and Baum, 1986). To accomplish this, the genes encoding the rRNA primary transcript are selectively amplified 100-1000-fold over somatic cells (Brown and Dawid, 1968; Gall, 1968). The amplified DNA forms large numbers of extrachromosomal nucleoli, which each contain one to twenty tandemly arranged rRNA genes (Brown and Dawid, 1968). Transcription from these amplified rRNA genes provides enough 18S, 28S and 5.8S rRNAs for the huge amounts of ribosomes that are synthesised in oocytes. However, 5S RNA must also be synthesised at a higher rate, to match that of the other rRNAs. The frog oocyte accomplishes this by having two sets of genes encoding 5S RNA. One encodes the 5S RNA found in somatic cells, and is present in 400 copies (Peterson *et al.*, 1980). The other encodes 5S RNA which is specifically found in oocytes, and is present in

20 000 copies (Peterson *et al.*, 1980). In somatic cells, the somatic-type 5S RNA genes are expressed, while the oocyte-type genes are repressed (reviewed in Wolffe, 1994). In the oocyte, the oocyte-type and somatic-type genes are both switched on, allowing 50 times more 5S RNA synthesis for ribosome stockpiling. The 5S RNA produced from the somatic-type and oocyte-type set of genes differs by six nucleotides (Ford and Southern, 1973).

1.3 PATHWAY TAKEN BY 5S RNA IN *XENOPUS* OOCYTES

Although the oocyte-type 5S RNA genes are switched on in oocytes, this only provides 50 times more templates for transcription, as compared with the 100-1000 times amplification of the DNA encoding the other rRNAs. Another mechanism is thus utilised in oocytes to provide necessary quantities of 5S RNA. This results in a pathway taken by 5S RNA that is more complicated in oocytes than the route taken in somatic cells. This pathway is described below, and is diagrammed in Figure 1-2.

Most ribosome assembly and stockpiling takes place during the vitellogenic stage of oogenesis. However, 5S RNA is synthesised before the other rRNAs and ribosomal proteins (Ford, 1971). In early oogenesis, 5S RNA is synthesised in fifteen to twenty-fold molar excess over the rRNAs, although this ratio decreases to three to four fold molar excess at later stages (Ford, 1971). In previtellogenic oocytes, this excess of 5S RNA is stored in the cytoplasm in two RNP particles, which sediment at 42S and 7S.

The basic unit of the 42S RNP consists of 5S RNA bound to a non-ribosomal protein, p43, transfer RNA (tRNA), and another non-ribosomal protein, p48, in a ratio of 1:1:3:2 (Ford, 1971; Picard *et al.*, 1980). The 42S RNP is a tetramer of this basic unit (Picard *et al.*, 1980). 42S RNPs are predominant in stages I and II of oogenesis, but are not detectable by stage VI (Dixon and Ford, 1982a,b; Viel *et al.*, 1990).

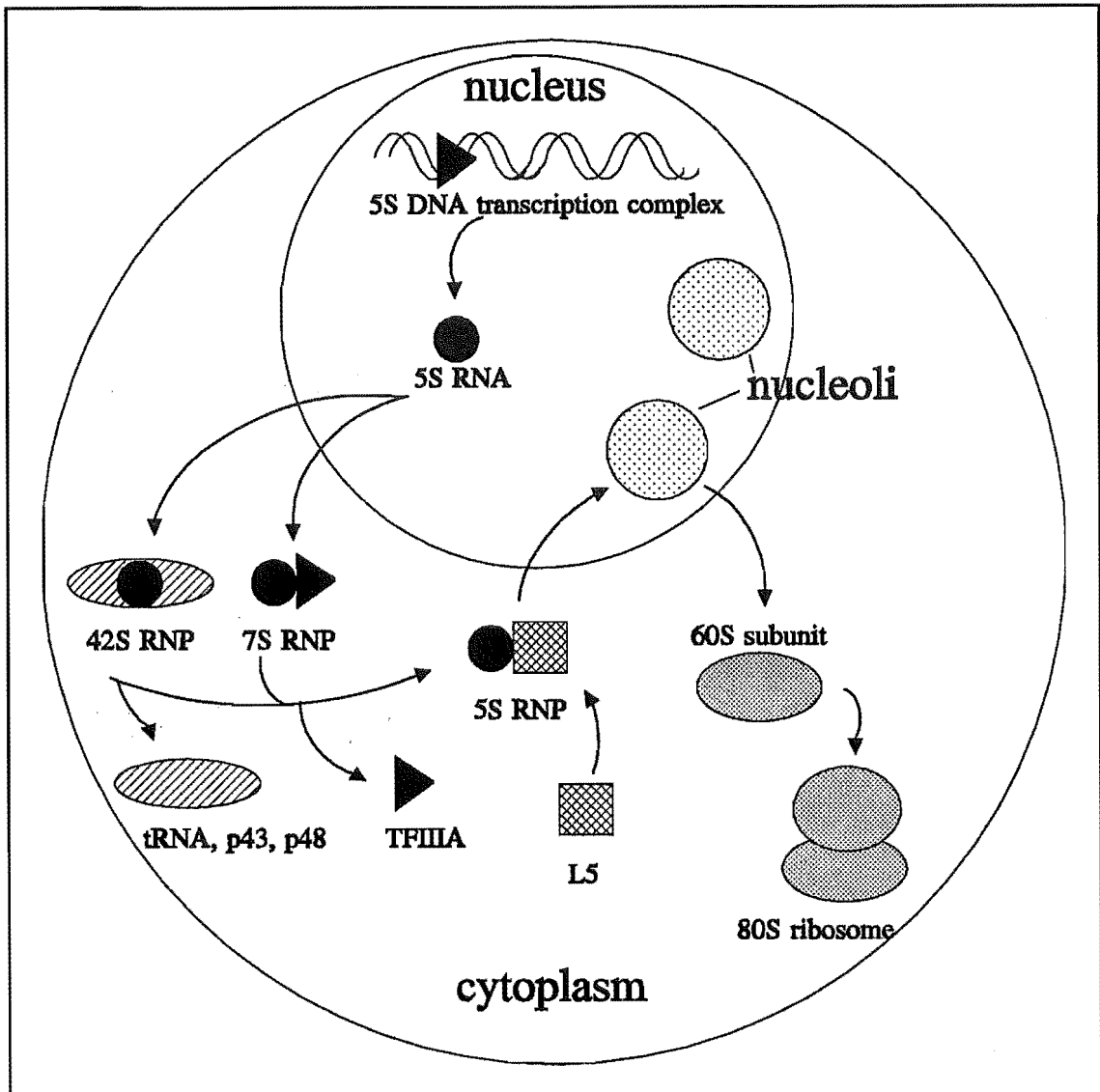


Figure 1-2 Pathway taken by 5S RNA in *Xenopus* oocytes.

The remaining 50% of 5S RNA is complexed in a one to one ratio with the protein TFIIIA (Picard and Wegnez, 1979; Honda and Roeder, 1980; Pelham and Brown, 1980). Thus, in oocytes, TFIIIA has the ability to bind both the 5S RNA gene and 5S RNA itself. TFIIIA is present in a constant amount (60 ng/oocyte) throughout the early stages of oogenesis, but declines twenty-fold by the end of oogenesis (Shastri *et al.*, 1984). Immunocytochemistry and immunoprecipitation assays have shown that both 42S and 7S RNPs are exclusively localised in the cytoplasm of oocytes (Mattaj *et al.*, 1983; Viel *et al.*, 1990; Allison *et al.*, 1991).

5S RNA is stored in 7S and 42S RNPs until vitellogenesis, when the other rRNAs and ribosomal proteins are synthesised. At this time 5S RNA is released from

the storage particles, and binds to ribosomal protein L5, forming 5S RNPs (Allison *et al.*, 1991). Synthesis of L5 is maximal during vitellogenesis, coinciding with synthesis of the rRNAs and other ribosomal proteins and ribosome assembly (Wormington, 1989). Since 5S RNPs were immunoprecipitated from both cytoplasmic and nuclear fractions of oocytes, L5 was implicated in the mobilisation of stored 5S RNA for import into the nucleus (Allison *et al.*, 1991). Once in the nucleus, 5S RNA is targeted to the amplified nucleoli, where it becomes incorporated into the 60S ribosomal subunit (Allison *et al.*, 1993). The final step of the 5S RNA pathway in oocytes is export from the nucleus to the cytoplasm as part of the 60S ribosomal subunit and storage in 80S monosomes for use in embryogenesis.

Although somatic-type 5S RNA is synthesised in oocytes (Ford and Southern, 1973), it was not detected in long term storage particles or ribosomes from vitellogenic oocytes (Denis and Wegnez, 1977). More recently, oocyte-type and somatic-type 5S RNAs were shown to have different behaviours after microinjection into the oocyte cytoplasm (Allison *et al.*, 1995). Oocyte-type 5S RNA predominantly associated with TFIIA, forming 7S RNP storage particles, whereas somatic-type 5S RNA preferentially associated with ribosomal protein L5. In addition, somatic-type 5S RNA was imported into the nucleus at a faster rate and to a larger extent, and more was incorporated into 60S ribosomal subunits (Allison *et al.*, 1995). These results further support the hypothesis that L5 mobilises 5S RNA for import into the nucleus and incorporation into ribosomal subunits.

The pathway taken by newly synthesised 5S RNA in late stage oocytes has also been investigated (Guddat *et al.*, 1990). When genes encoding 5S RNA were microinjected into oocyte nuclei, the transcribed RNA transiently associated with the La antigen (Guddat *et al.*, 1990). The La protein was then replaced by either TFIIA or L5, and these RNPs exported to the cytoplasm. 5S RNA molecules impaired in their ability to bind L5 and TFIIA were retained in the nucleus (Guddat *et al.*, 1990).

1.4 THE PRESENT STUDY

The pathway taken by 5S RNA in oocytes of *Xenopus laevis* involves multiple RNA-protein interactions, bi-directional nuclear transport, and nucleolar targeting. Although the overall pathway is known, the molecular interactions and mechanisms of regulation for each step remain to be elucidated. In the present study, I have investigated some of the steps taken in this pathway in detail, including storage of 5S RNA as a 7S RNP in the cytoplasm of previtellogenic oocytes, targeting of 5S RNA to the nucleolus, and incorporation into the 60S ribosomal subunit.

Chapter 2 presents the methods used in this investigation. Chapter 3 describes experiments in which the specific sequence and structural requirements of 5S RNA for protein associations in the cytoplasm and for ribosomal incorporation were investigated, using a series of mutant 5S RNA molecules. Chapter 4 describes experiments in which the intranuclear distribution of endogenous and exogenous mutant 5S RNA molecules was examined to determine the requirements of 5S RNA for nucleolar localisation. Chapter 5 addresses the possible mechanisms for the retention of 7S RNPs in the cytoplasm of previtellogenic oocytes, in particular, the role of cytoskeletal and intracellular membrane structures.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Chemicals used in this study were purchased from BDH (BDH Chemicals New Zealand Ltd., Palmerston North, New Zealand), unless otherwise specified. Restriction enzymes were purchased from Boehringer Mannheim N. Z. Ltd. (Auckland, New Zealand). All solutions were made using ultrafiltered H₂O from a Barnstead Nanopure Ultrapure water system. *Xenopus laevis* were either bred in the Department of Zoology at the University of Canterbury, or were purchased from Xenopus I (Ann Arbor, Michigan, USA).

I. Plasmids

DNA templates for the synthesis of wild-type and mutant 5S RNA molecules were a generous gift from P. J. Romaniuk (University of Victoria, Victoria, British Columbia, Canada). Plasmids contain one copy of a 5S RNA gene, constructed from a series of synthetic oligonucleotides which were subsequently annealed and ligated into the vector pUC18 (Romaniuk *et al.*, 1987a). Some of these clones were subsequently inserted into M13mp18 (Baudin and Romaniuk, 1989). The 5S RNA

genes were constructed so that digestion with *Dra*I and transcription with T7 RNA polymerase yields a 121 nucleotide molecule with identical 5' and 3' termini to those found in naturally occurring 5S RNA molecules. The U1 snRNA template (Hamm *et al.*, 1987) was provided by D. S. Goldfarb (University of Rochester, Rochester, New York, USA). After linearisation with *Bam* HI and transcription with T7 RNA polymerase, this synthetic U1 snRNA contains three additional G residues at the 5' end and four additional nucleotides (GATC) at the 3' end (Hamm *et al.*, 1987). The U3 snoRNA gene template (pXIU3A'; Savino *et al.*, 1992) was provided by S. A. Gerbi (Brown University, Providence, Rhode Island, USA). The template used for transcription of U3 snoRNA was produced by M. Ezrokhi (Brown University), using PCR as described in Terns and Dahlberg (1994). The template contains a T7 promoter and produces U3 snoRNA with 5 extra nucleotides at the 3' end (3'-UUUUA), which enhances the *in vivo* stability of the transcript.

pSP6-L5b containing the *Xenopus* ribosomal protein L5 cDNA clone (Wormington, 1989) was provided by W. M. Wormington (University of Virginia, Charlottesville, Virginia, USA).

Plasmid pXlo8G contains one repeat unit of oocyte-type 5S DNA from pXlo8 subcloned into pGEM4 (Allison *et al.*, 1991). Transcription of pXlo8 linearised with *Sma*I with T7 RNA polymerase yields a transcript of 714 nucleotides containing an antisense 5S RNA sequence. Plasmid pSP72-Vg1 was a generous gift from D. A. Melton (Harvard University, Cambridge, Massachusetts, USA). This plasmid contains a fragment of *Xenopus* Vg1 cloned into the *Sma*I site of pSP72. Digestion with *Eco* RI and transcription with SP6 RNA polymerase produces a transcript containing an antisense Vg1 sequence. Plasmid pXlr101A was a gift from A. H. Bakken (University of Washington, Seattle, Washington, USA), and contains a full repeating unit of the *Xenopus* ribosomal DNA (rDNA) cloned into the *Hind* III site of pBR322 (Bakken *et al.*, 1982).

II. Antisera

Anti-TFIIIA and anti-60S ribosomal subunit antisera were generously provided by M. le Maire (CEA et CNRS, Gif-sur-Yvette, France). These antisera are described and characterised in Viel *et al.* (1990) and Allison *et al.* (1993). Anti-NO38 (No-185; Schmidt-Zachmann *et al.*, 1987) was generously donated by M. S. Schmidt-Zachmann (German Cancer Research Centre, Heidelberg, Germany). Anti-*Artemia* L5 was provided by N. Kenmochi (Niigata University School of Medicine, Niigata, Japan), and is described in Kenmochi and Ogata (1989).

2.2 IN VITRO TRANSCRIPTION OF RNA

I. ³²P-labelled 5S RNAs

³²P-labelled wild-type or mutant 5S RNAs were produced from gene templates linearised with *Dra*I, using one of the following two methods.

i. Method A

A final transcription reaction volume of 20 µl contained 40 mM Tris-HCl, pH 8.1 @ 37°C, 15 mM MgCl₂, 5 mM dithiothreitol (DTT), 0.1 mg/ml Bovine Serum Albumin (BSA; Boehringer Mannheim), 1 mM spermidine, 40 U RNasin (Promega: Pacific Diagnostics, Pty. Ltd., Auckland, New Zealand), 1 mM each of ATP, CTP, and UTP, and 0.025 mM GTP (Boehringer Mannheim), 1 µg linearised template DNA, 50-100 µCi [α-³²P]GTP (3000 Ci/mmol; Amersham Australia Pty. Ltd., Auckland, New Zealand) and 20 U T7 RNA polymerase (Boehringer Mannheim). The reaction was incubated at 37°C for 1.5 hr. The DNA template was removed by treatment with 1 U RNase-free DNase I (Boehringer Mannheim) for 15 min at 37°C.

Samples were extracted once with an equal volume each of phenol (saturated in TE [10 mM Tris-HCl, 1 mM EDTA], pH 8.0) and chloroform:isoamyl alcohol

(24:1; hereafter referred to as "chloroform"), and then once with an equal volume of chloroform. RNA was precipitated two times, each with 2.5 M ammonium acetate and 2 volumes of 100% ethanol. RNA pellets were resuspended in TE pH 7.6, aliquoted and stored at -80°C .

RNA was quantified using DNA Dipstick (Invitrogen Corp., San Diego, California, USA). Typical yields were between 50-100 ng of RNA.

ii. Method B

Transcription reactions contained T7 transcription buffer (Epicentre Technologies: Intermed Scientific Ltd., Auckland, New Zealand), 10 mM DTT, 40 U RNasin, 200 μM CTP, ATP, and UTP, 40 μM GTP, 1 μg linearised template DNA, 50-100 μCi [α - ^{32}P]GTP (3000 Ci/mmol; DuPont NEN: Life Technologies Ltd., Auckland, New Zealand) and 50 U T7 RNA polymerase (Epicentre). The 20 μl mixture was incubated at 37°C for 1 hr followed by treatment with 1 U RNase-free DNase. One hundred and twenty-eight microlitres of UB Blue (175 mM NaCl, 5 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.5% sodium dodecyl sulphate [SDS], 0.05% methylene blue, 7 M urea) and 20 μg glycogen (Boehringer Mannheim) were added, and the sample extracted once with an equal volume of phenol/chloroform and once with an equal volume of chloroform. The RNA was precipitated with 2 volumes of 100% ethanol on ice for 15 min, and again with 2.5 M ammonium acetate and 2 volumes of 100% ethanol on ice for 15 min. The RNA was resuspended in TE, pH 7.6 and stored at -80°C .

RNA was quantified using DNA Quik STRIP (Eastman Chemical Co., New Haven, Connecticut, USA). Typical yields were approximately 300 ng. ^{32}P -labelled RNA was also counted in a Quick-Count QC-2000 benchtop radioisotope counter (Bioscan Inc., Washington, D. C., USA) to allow comparable dilutions of the different RNAs.

II. ³²P-labelled U1 snRNA and U3 snoRNA

Reactions were performed as described in Method B for 5S RNA, containing 1 µg of U1 or U3 template, with the addition of 0.4 mM m⁷G cap (New England Biolabs) to the reaction mixture. The RNAs were purified as in Method B above and stored at -80°C.

III. ³³P-labelled RNAs

For *in situ* localisation assays, RNAs were synthesised containing [³³P]UTP, as this isotope has five-fold weaker β-emissions than ³²P, allowing higher autoradiographic resolution. RNAs were synthesised in a reaction containing 200 µM CTP, ATP, and GTP, 40 µM UTP and 50 µCi [α-³³P]UTP (1000-3000 Ci/mmol; Amersham) and purified as described in Method B above.

IV. DIG-labelled Antisense RNA Probes

For northern hybridisation, digoxigenin (DIG)-labelled antisense RNAs were synthesised from pXlo8G and pSP72-Vg1. DIG-labelled sense-strand 5S RNA probes were synthesised using the pXlo-wt template. Twenty microlitre reaction mixtures contained 40 mM Tris-HCl, pH 8.0, 6 mM MgCl₂, 10 mM NaCl, 10 mM DTT, 2 mM spermidine, 40 U RNasin, 1 mM each of ATP, GTP and CTP, 0.65 mM UTP, 0.35 mM DIG-11-UTP (Boehringer Mannheim), 1 µg template DNA and 40 U T7 RNA polymerase (for antisense and sense-strand 5S RNA; Epicentre) or SP6 RNA polymerase (for antisense Vg1; Boehringer Mannheim). Reactions were incubated for 1.5 hr at 37°C, and the DNA digested with DNase. RNA was purified as described in Method A above, dissolved in TE, pH 7.6, and stored at -80°C.

The yield of DIG-labelled RNA was estimated by spotting serial dilutions of transcripts onto a nylon membrane followed by visualisation using DIG chemiluminescent detection (see section 2.10.IV) and comparison with DIG-labelled

RNA standards (Boehringer Mannheim). Typical transcription yields were estimated to be 10-20 µg RNA.

2.3 *IN VITRO* SYNTHESIS OF ³⁵S-LABELLED L5

³⁵S-labelled ribosomal protein L5 was synthesised *in vitro* using a rabbit reticulocyte coupled transcription-translation system for templates with SP6 promoters (Promega). Reactions were performed following the manufacturer's instructions, with 1 µg of non-linearised pSP6-L5b, containing 50 µCi [³⁵S]methionine (>1000 Ci/mmol; DuPont NEN), and incubated at 30°C for 1.5 hr. Reaction mixtures were then filtered through a 0.22 µm filtration unit (Millipore: BioLab Scientific, Christchurch, New Zealand) to remove large aggregates before microinjection. Labelled protein was aliquoted and stored at -80°C.

2.4 OOCYTE ISOLATION AND PREPARATION

Mature *Xenopus laevis* females were anaesthetised on ice, and lobes of ovary surgically removed through a small abdominal incision, which was then sutured. Ovarian tissue was rinsed three times in 0.15 M NaCl, 0.05 M Tris-HCl, pH 8.0, and 1 mM EDTA to remove the blood, then rinsed twice in phosphate buffered saline (PBS; 68 mM NaCl, 1.3 mM KCl, 4.0 mM Na₂HPO₄, 0.7 mM KH₂PO₄, 0.35 mM CaCl₂, 0.25 mM MgCl₂) to remove the EDTA. The tissue was then digested with 1 mg/ml collagenase (Type II, Sigma, St. Louis, Missouri, USA) diluted in 0.1 M sodium phosphate pH 7.4, at room temperature with end over end rotation for 20-30 min. Oocytes were then rinsed twice with PBS and twice with O-R2 (82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1.0 mM Na₂HPO₄, 3.8 mM NaOH, 5.0 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid], pH 7.8; Wallace *et al.*, 1973), and incubated in O-R2 at 18°C. All procedures used were approved by the University of Canterbury Animal Ethics Committee.

Oocytes were separated into stages according to Dumont (1972) using the following criteria: stage I, transparent, 50-300 µm; stage II, white/opaque,

300-400 μm ; stage III, light brown/tan, 450-600 μm ; stage IV, animal and vegetal hemispheres differentiated, animal pole dark brown, 600-1000 μm ; stage V, animal pole brown/beige colour, 1000-1200 μm ; stage VI, unpigmented equatorial band, 1200-1300 μm .

2.5 MICROINJECTION

I. Equipment

A PW-6 microelectrode puller (Narashige, Tokyo, Japan) was used to pull 1 mm O. D. glass needles (Clark Electromedical Instruments, Reading, England). The microinjection equipment consisted of a micromanipulator and PV 830 Pneumatic PicoPump (World Precision Instruments, Inc., New Haven, Connecticut, USA), a Schott KL1500 cold light source, and a Heerbrugg stereo-microscope. Vacuum was provided by a Gast oil-less diaphragm vacuum pump, and pressure by a tank of N_2 gas. Microinjection dishes were made by gluing Nitex 900 μm mesh to culture dishes with chloroform.

II. Procedure

Microinjection needles were pulled and ground to a 20 μm tip. Solutions to be injected were loaded into the needle using approximately 25 mm Hg vacuum. The injected volume was calibrated using the drop diameter and adjusted by regulating the pulse time. Healthy stage V oocytes were transferred to microinjection dishes in O-R2 media, and injections were performed into the vegetal hemisphere. After injection, oocytes were placed on ice for 30 min to aid wound healing, and then incubated at 18°C for specified lengths of time.

2.6 IMMUNOPRECIPITATION ASSAYS

To analyse the incorporation of wild-type and mutant 5S RNAs into oocyte RNPs, immunoprecipitation assays were performed on either whole oocytes or nuclear fractions using antibodies specific for these RNPs. This technique utilises the high affinity of protein A for immunoglobulins, which, when attached to a solid matrix allows the purification of antibody-antigen complexes.

I. Whole Oocytes

Forty to eighty nanolitres of ^{32}P -labelled wild-type or mutant 5S RNAs (0.2 to 0.5 ng RNA), synthesised using Method A, were injected into the cytoplasm of oocytes which were then incubated for 48 hr. Protein A-Sepharose (CL-4B; Pharmacia LKB Biotechnology, Auckland, New Zealand) was swollen in Ipp150 (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% NP-40 [nonidet P40]) at 5 mg/ml for 15 min, then 500 μl aliquots were incubated for 2 hr with end over end rotation with 10 μl anti-TFIIIA or anti-60S ribosomal subunit antisera to allow binding of antibodies to protein A. The resin was then pelleted for 10 s in a microfuge and resuspended in 1 ml of Ipp150. This wash was repeated three times to remove all unbound antibody.

Whole oocytes were homogenised in Ipp150 containing 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 10 U/ml RNasin using a Gilson p1000 Pipetman, and cleared of yolk and pigment by centrifugation at 10 000 rpm for 10 min at 4°C. Supernatants were divided into 500 μl samples (20 oocyte equivalents) and aliquoted onto protein A-Sepharose-antibody pellets, and incubated for 1 hr at 4°C with end over end rotation. The resin was then pelleted and the immunosupernatants kept. Pellets were washed four times with Ipp150 to remove unbound cellular material. After the final wash, 300 μl of Ipp150 was added to each pellet, followed by 2 μg of carrier yeast tRNA (Gibco BRL: Life Technologies), 30 μl of 10% SDS and an equal volume of phenol/chloroform. RNA was also extracted from immunosupernatants by adding 50 μl of 10% SDS and an equal volume of phenol/chloroform. The samples were vortexed and incubated for 15 min

at 37°C with frequent mixing. The aqueous phase was recovered after centrifugation, and the RNA precipitated with 0.4 M sodium acetate and ethanol. RNA pellets were dissolved in 10-20 µl of formamide-dye loading buffer (95% deionised formamide, 0.05% xylene cyanol, 0.05% bromophenol blue), and denatured by boiling for 3 min.

Samples were resolved by denaturing electrophoresis on 8% polyacrylamide/8 M urea gels in TBE running buffer (89 mM Tris, 89 mM boric acid, 2.7 mM EDTA) for 1 hr at 650 V (0.75 mm x 20 cm gels). Gels were dried for two hours at 80°C in a BioRad model 583 gel dryer (BioRad Laboratories Pty. Ltd., Auckland, New Zealand) with vacuum supplied by water pressure. Dried gels were exposed to X-ray film (Hyperfilm-MP, Amersham) with intensifying screens at -80°C for overnight (immunosupernatants) or 3 weeks (immunoprecipitates). Films were developed in AGFA G150 developer for 3 min and fixed in Ilford Hypam rapid fixer for 4 min.

For characterisation of antisera, endogenous oocyte RNA was labelled by microinjection of 40 nl of 10 mCi/ml [³²P]GTP (DuPont NEN) into the cytoplasm of oocytes which were incubated overnight. Homogenates of 20 oocytes were prepared as above for immunoprecipitation. For control experiments, oocyte homogenates (containing no PMSF) were treated with 200 µg/ml proteinase K (Boehringer Mannheim) for 1 hr at 37°C. This reaction was stopped with the addition of 10 mM EDTA. Immunoprecipitations were then performed as described above.

II. Nuclear Fractions

For immunoprecipitation of oocyte nuclei, oocytes were cytoplasmically injected with 20 nl of ³²P-labelled 5S RNA (synthesised using Method B) and incubated for 18 hr. Nuclei were dissected using watchmaker's forceps in nucleus isolation buffer (25 mM Tris-HCl, pH 8.0, 10% glycerol, 5 mM MgCl₂, 2 mM DTT; McKnight *et al.*, 1980). Immunoprecipitation assays were performed as described above, with the following modifications: Assays were performed in Ipp300 (50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 0.05% NP-40), using 20 µl of anti-TFIIIA, anti-L5 or anti-60S subunit antisera. As a control, immunoprecipitations were performed using 20 µl of normal rabbit serum (Sigma). For sequential immunoprecipitations,

immunosupernatants from the first precipitation were added to a second protein A-Sepharose-antibody pellet and incubated a further hour at 4°C with end over end rotation. To prevent degradation, fresh PMSF and RNasin were added to the second immunoprecipitation reaction. The RNA was extracted from immunoprecipitates and immunosupernatants and analysed as described for whole oocytes.

2.7 NON-DENATURING GEL ELECTROPHORESIS ASSAYS

For non-denaturing gel electrophoresis assays of mutant 5S RNAs, oocytes were cytoplasmically injected with 20-40 nl ^{32}P -labelled RNA, synthesised using Method A, and incubated for 48 hr. Three to five oocytes were homogenised with a Gilson p20 Pipetman in 20 μl RNP homogenisation buffer (20 mM Tris-HCl, pH 7.6, 100 mM KCl, 1.5 mM MgCl_2 , 2 mM DTT, 0.1 mM PMSF, 10 U/ml RNasin) and centrifuged for 5 min at 10 000 rpm at 4°C to remove yolk and pigment. One-tenth volume of 10 X glycerol dye loading buffer (200 mM EDTA, 50% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) was added to supernatants, which were then resolved on 6% polyacrylamide gels containing 0.1% Triton X-100, in TBE running buffer containing 0.1% Triton X-100, for 1.5 hr at 300 V (0.75 mm x 20 cm gel). Gels were dried for 30 min at 80°C under vacuum, and exposed to X-ray film at -80°C for 1-20 days.

For analysis of nuclear fractions, 20 nuclei were dissected in RNP homogenisation buffer without PMSF and RNasin, and then homogenised and analysed as described above. Nuclear fractions treated with proteinase K were incubated at 37°C for 30 min with 50 $\mu\text{g}/\text{ml}$ proteinase K. For EDTA treatment, nuclei were dissected and homogenised in RNP homogenisation buffer containing 25 mM EDTA.

2.8 NUCLEOLAR LOCALISATION ASSAYS

For analysis of the nucleolar localisation of 5S RNA mutants, 20 nl of either ^{32}P - or ^{33}P -labelled RNA (0.1 to 1 ng RNA) was microinjected into the cytoplasm of oocytes, which were incubated overnight. For nucleolar localisation of ^{35}S -labelled ribosomal protein L5, 50 nl of filtered L5 lysate reaction mixture was injected into oocytes which were incubated overnight in O-R2 containing 100 $\mu\text{g/ml}$ cycloheximide to prevent incorporation of excess [^{35}S]methionine into oocyte proteins. Nucleolar localisation was analysed by the following two methods.

I. Biochemical Fractionation

Nucleoli were isolated using the method of Peculis and Gall (1992). Nuclei were dissected from stage V oocytes in Nucleolar Isolation Buffer (83 mM KCl, 17 mM NaCl, 6.5 mM Na_2HPO_4 , 10 mM MgCl_2 , 1 mM EDTA, 1 mM DTT) using watchmaker's forceps, and collected in microfuge tubes. Isolated nuclei were sonicated in approximately 50 μl Nucleolar Isolation Buffer in a Branson bath sonicator (Bransonic 2) for 20 s in ice-water. Samples were centrifuged for 20 min at 13 000 rpm in a bench top microfuge to pellet nucleoli. After drawing off the supernatant (nucleoplasmic fraction), nucleolar pellets were washed by adding 200 μl Nucleolar Isolation Buffer, centrifuging for 5 min and discarding the supernatant.

i. RNA extraction and analysis

RNA was extracted from oocyte fractions using a procedure modified from Peppel and Baglioni (1990). Fractions were homogenised in 500 μl of Solution 1 (2% SDS, 200 mM Tris-HCl, pH 7.5, 1 mM EDTA) and vortexed for 5 s at high speed. One hundred and fifty microlitres of cold Solution 2 (42.9 g potassium acetate, 11.2 ml acetic acid and water to 100 ml) was added, and mixed by vortexing at medium speed for 10 s. Proteins and DNA were precipitated by placing on ice for 2 min and centrifuging 5 min at 13 000 rpm. Supernatants were extracted twice with 300 μl of chloroform, and the RNA precipitated with 650 μl of isopropanol.

Samples were resuspended in 10 µl formamide-dye loading buffer and denatured by boiling for analysis by 8 M urea/8% polyacrylamide gel electrophoresis (PAGE) and autoradiography. A Zeineh analytical hand-held scanning densitometer and Biomed Image Analysis software (Advanced American Biotechnology, Fullerton, California, USA) were used to quantify the intensity of bands on suitable exposures of autoradiograms (within the linear range of signal intensity of the film). Alternatively, samples were resuspended in 50 µl of TE, pH 7.6 and added to 2 ml of Biodegradable Counting Scintillant (BCS; Amersham) for direct counting of samples in a scintillation counter (Beckman LS 2800).

ii. Protein extraction and analysis

Nucleolar pellets were dissolved directly in 10 µl SDS-PAGE sample buffer (2% SDS, 100 mM DTT, 60 mM Tris-HCl, pH 6.8, 0.01% bromophenol blue). Nucleoplasmic proteins were precipitated with 5 volumes of acetone overnight at -20°C, centrifuged at 13 000 rpm for 10 min, and resuspended in 20 µl SDS-PAGE sample buffer. Samples were boiled for 10 min and placed on ice prior to electrophoresis. Proteins were separated on discontinuous 12% polyacrylamide gels containing 0.1% SDS in SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) for 4 hr at 200 V (0.75 mm x 20 cm gel). After electrophoresis, gels were fixed for 30 min in 25% isopropanol, 10% acetic acid and 3% glycerol, followed by soaking in Amplify (Amersham) for 30 min. Gels were air dried on perspex gel drying frames with a cellophane cover (Tut's Tomb, Idea Scientific Co.), then exposed to X-ray film at -80°C for 2 weeks.

II. *In Situ* Localisation

i. Preparation of sections

Oocytes were fixed for 24 hr in 5% acetic acid, 2% formaldehyde, 250 mM NaCl, after which an equal volume of 95% ethanol was added dropwise with mixing

over 15 min, followed by a further 15 min incubation (Allison *et al.*, 1991). The oocytes were dehydrated further in 70% ethanol for 30 min, 90% ethanol for 30 min, and 2 changes of 100% ethanol for 15 min each. They were then incubated in Cedarwood oil (Gurr) for 2-6 hr with gentle rotation for clearing. Oocytes were infiltrated with paraffin wax by incubating in three changes of molten wax at 65°C under vacuum for 5 min each. The oocytes were embedded in paraffin wax in watch glasses. The wax was hardened overnight, and the blocks trimmed to trapezoids, and sectioned using a Beck microtome at 4-7 μm .

Slides were prepared by soaking in 1 N HCl overnight, rinsing with tapwater and distilled water and then air dried. Subbing solution was prepared by dissolving 0.1% gelatin (Sigma) by heating, then adding 0.01% chromic potassium sulphate and filtering through a 45 μm pore membrane. Slides were dipped into subbing solution, drained and baked at 65°C overnight. Sections were adhered onto subbed slides by floating in sterile H_2O , which was heated to approximately 45°C to decompress the sections. The H_2O was gently drawn off and the slides dried overnight at 37°C.

ii. Autoradiography and staining

Paraffin was removed from sections by two changes of xylene for 3 min each, followed by a rinse in 1:1 xylene:ethanol, then 100% ethanol for 3 min. The slides were then air dried.

LM-1 emulsion (Amersham) was melted in the dark at 43°C for 15 min, then diluted 1:1 with sterile distilled H_2O , also at 43°C. Slides were slowly dipped into diluted emulsion for 5 s, drained and air dried for 2 hr, sealed in light-proof racks containing silica crystals and exposed at 4°C for 4 days to 4 weeks.

Before developing, slides were warmed to room temperature for 2 hr to prevent condensation forming. Slides were developed at 18°C in D19 developer (Kodak) for 5 min, washed for 30 s in 0.5% acetic acid, fixed for 10 min in Ilford Hypam fixative, and rinsed for 15 min in running tapwater and twice in distilled H_2O . Giemsa stain was prepared by adding 2 ml of Giemsa stock (Gurr: BDH; 0.5 g Giemsa dissolved in 33 ml glycerol and 33 ml methanol) to 200 ml of 3.35 mM Na_2HPO_4 and 3.35 mM KH_2PO_4 . Sections were stained for 10 min at room temperature, thoroughly rinsed with distilled H_2O and air dried. Slides were viewed

under brightfield microscopy using an Olympus BH-2 microscope and photographed with either Agfacolor Optima 125 print film using neutral density and blue filters, or with Fujichrome 64T slide film using a neutral density filter.

2.9 WESTERN ANALYSIS

I. Preparation of Samples

Total oocyte proteins were prepared by homogenising a mixture of 10 mature and 20 immature oocytes in cold 100 mM NaCl, 20 mM Tris-HCl, pH 7.6 and 0.1 mM PMSF, and centrifuging at 4°C for 10 min at 10 000 rpm. Supernatants were diluted with 2 X SDS-PAGE sample buffer for electrophoresis.

5S RNPs were purified from EDTA-treated ribosomes isolated from ovaries of mature *Xenopus* females by L. A. Allison as previously described (Allison *et al.*, 1991). For western analysis, 2 µl of purified sample was diluted with SDS-PAGE sample buffer.

7S RNPs were prepared using a modified protocol of Blanco *et al.* (1989). Ovaries were removed from five immature *Xenopus* females (containing stage I-III oocytes) and washed three times in 0.15 M NaCl, 0.05 M Tris-HCl, pH 8.0, 1 mM EDTA and twice in homogenisation buffer (60 mM NH₄Cl, 7 mM MgCl₂, 10 mM HEPES, pH 7.5, 25 mM DTT, 0.1 mM PMSF). Tissue was homogenised in 3 ml cold homogenisation buffer with a glass homogeniser and centrifuged at 2°C in a Sorvall SS-34 rotor at 12 000 x g for 10 min. The supernatant was recovered and loaded onto a DEAE-Sepharose (CL-6B; Sigma) column pre-equilibrated with Buffer A (20 mM HEPES, pH 7.5, 0.1 mM EDTA, 5 mM MgCl₂, 10% glycerol, 100 mM KCl, 0.5 mM DTT). The column was then washed with 5 column volumes of buffer A containing 150 mM KCl. 7S RNPs were eluted with 5 ml buffer A containing 320 mM KCl, and stored at -80°C. For western analysis, 8 µl of the 7S RNP preparation was diluted with SDS-PAGE sample buffer.

For western analysis of endogenous nucleolar and nucleoplasmic proteins, nuclei from 50 stage V oocytes were separated into nucleolar and nucleoplasmic

fractions as described in section 2.8.I. Nucleolar pellets were dissolved directly in SDS-PAGE sample buffer. Nucleoplasmic fractions were precipitated with 5 volumes of cold acetone on ice for 10 min and centrifuged for 5 min at 5000 rpm to pellet the proteins. Pellets were dissolved in SDS-PAGE sample buffer.

II. Electrophoresis and Transfer

Prior to electrophoresis, samples were boiled for 10 min and placed immediately on ice. Proteins were separated on discontinuous 12% polyacrylamide gels containing 0.1% SDS in SDS running buffer at 200 V for 1 hr (1 mm x 7 cm mini gel). Proteins were transferred to nitrocellulose membrane (Hybond-C, Amersham) using a BioRad Mini-Trans Blot electrophoretic transfer apparatus in 25 mM Tris, 190 mM glycine and 20% methanol for 18 hr at 30 V (approximately 48 mA) at 4°C, according to the manufacturer's instructions. After transfer, membranes were stained with 0.2% Ponceau S (from a 2% stock dissolved in 3% trichloroacetic acid [TCA] and 3% sulphosalicylic acid) for 5-10 min to confirm transfer and to stain high molecular weight protein standards (BioRad).

III. Immunodetection

Immunodetection was performed using a protocol from Harlow and Lane (1988) for antibodies conjugated to horseradish peroxidase (POD). Membranes were thoroughly rinsed in PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.2) to remove Ponceau S stain. Membranes were blocked for 2 hr at room temperature in 3% BSA (fraction V; Boehringer Mannheim) in PBS (BSA/PBS) followed by two 5 min rinses in PBS. Primary antibodies (*Artemia* anti-L5 or anti-NO38) were diluted 1:1000 in 3% BSA/PBS before incubation for 1 hr, followed by four 5 min washes in PBS. Membranes were then incubated in either anti-rabbit (for L5 detection; Boehringer Mannheim) or anti-mouse (for NO38 detection; Vector Laboratories) IgG-POD secondary antibodies diluted 1:2000 in 3% BSA/PBS for 1 hr, followed by four washes for 5 min in PBS. For detection of proteins, a 30 mg/ml stock solution of the POD substrate, 4-chloro-1-naphthol

(dissolved in ethanol; Sigma) was diluted 1:100 in 50 mM Tris-HCl, pH 7.6 and filtered through Whatmann No. 1 filter paper. Membranes were incubated in 10 ml of this chloronaphthol solution containing 10 µl of 30% hydrogen peroxide until bands were suitably dark (5-30 min). The reaction was stopped by rinsing with PBS.

2.10 NORTHERN ANALYSIS

I. Preparation of Samples

For the analysis of nucleolar localisation of endogenous 5S RNA and other rRNAs, RNA was extracted from nucleolar and nucleoplasmic fractions from 40 nuclei as described in section 2.8.I.

RNA samples from oocytes fractionated into cytoskeletal and membrane fractions were prepared as described in sections 2.11 and 2.12, respectively.

Samples were resuspended for electrophoresis in TBE with 14% formaldehyde and denatured at 65°C for 15 min. After heating, samples were placed immediately on ice and 0.1 volumes each of 10 X glycerol-dye loading buffer and of a 1 mg/ml solution of ethidium bromide were added.

II. Electrophoresis and Transfer

RNA samples were separated on either 1.8% agarose (for analysis of 5S RNA and Vg1) or 1% agarose (for analysis of rRNAs) gels (without formaldehyde; Liu and Chou, 1990) in TBE for 2-3 hrs at 60-80 V. RNA was transferred by capillary transfer to positively charged nylon membrane (Boehringer Mannheim) in 20 X SSC (3 M NaCl, 0.3 M sodium citrate) overnight as described in Sambrook *et al.* (1989). After transfer, membranes were soaked in 6 X SSC for 5 min, then placed between two pieces of 3 MM paper (Whatmann). RNA was immobilised on filters by exposure to ultraviolet irradiation for 3 min.

III. Hybridisation

i. Antisense RNA probes

DIG-labelled antisense 5S RNA and Vg1 RNA probes were synthesised as described in section 2.2.IV. Membranes were prehybridised 2-4 hr at 68°C in 20-40 ml hybridisation buffer (50% formamide, 5 X SSC, 2% blocking solution [see section IV; Boehringer Mannheim], 0.1% sarkosyl, 0.02% SDS). This solution was then removed and replaced with 10 ml fresh hybridisation buffer preheated to 68°C containing 50-200 ng/ml DIG-labelled RNA probe which had been denatured by heating in hybridisation buffer at 68°C for 15 min. Membranes were hybridised overnight at 68°C with agitation. Subsequently, membranes were washed twice in 2 X SSC and 0.1% SDS at room temperature for 5 min each, and twice in 0.1 X SSC and 0.1% SDS at 68°C for 15 min each, with agitation.

ii. DNA probes

DIG-labelled rDNA probes were synthesised from 2 µg of denatured pXlr101A digested with *Hind* III using a random priming reaction containing 100 U/ml Klenow enzyme, hexanucleotide mixture and dNTP labelling mixture, at 37°C overnight, according to the manufacturer's instructions (Boehringer Mannheim). Reactions were stopped with the addition of 20 mM EDTA and the DNA was precipitated with 0.4 M LiCl and 3 volumes of ethanol. After centrifugation, DNA pellets were dissolved in TE, pH 8.0 and stored at -20°C. The yield of DIG-labelled probe was estimated by spotting serial dilutions onto a nylon membrane, followed by visualisation using DIG chemiluminescent detection (see section IV) and comparison with DIG-labelled control DNA (Boehringer Mannheim). Total yields of DIG-labelled DNA were estimated to be 1 µg.

Membranes were prehybridised for 4 hr at 50°C in 20-40 ml SDS hybridisation buffer (50% formamide, 5 X SSC, 2% blocking solution [see section IV; Boehringer Mannheim], 50 mM sodium phosphate, pH 7.0, 7% SDS, 0.1% sarkosyl, 50 µg/ml yeast tRNA). The prehybridisation solution was then

removed, and replaced with 10 ml fresh SDS hybridisation buffer preheated to 50°C, containing 5 ng/ml DIG DNA probe denatured by boiling. Membranes were hybridised overnight at 50°C with agitation. Subsequently, membranes were washed as described for RNA probes (section III.i).

IV. DIG Chemiluminescent Detection

DIG detection was performed using reagents purchased from Boehringer Mannheim, following the manufacturer's instructions. All steps were performed at room temperature unless otherwise indicated, and with agitation. Membranes were initially washed 5 min in 0.3% Tween 20 in Buffer 1 (0.1 M maleic acid, 0.15 M NaCl, pH with NaOH to 7.5) then incubated for 30 min in 1% blocking solution (Blocking reagent dissolved in Buffer 1). Membranes were then incubated with anti-digoxigenin Fab fragments conjugated to alkaline phosphatase, diluted 1:10 000 in 1% blocking solution for 30 min, followed by two washes in 0.3% Tween 20 in Buffer 1 for 15 min each. Membranes were then equilibrated for 5 min in Buffer 3 (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl), then incubated for 5 min in substrate solution (AMPPD or Lumigen PPD) diluted 1:100 in Buffer 3. Membranes were blotted for a few seconds on 3 MM paper, sealed in Glad Wrap and incubated for 15 min at 37°C before exposure to X-ray film at room temperature for 1 min to 2 hr.

2.11 CYTOSKELETON PREPARATION

Cytoskeletal fractions of oocytes were prepared using four different extraction buffers, each of which is described below. The first was the method of Yisraeli *et al.* (1990), which was designed to isolate the oocyte cytoskeleton free of yolk proteins. The other three procedures were designed to enrich specific components of the cytoskeleton.

I. General Procedure

Ten mature (stage V) or 20 immature (stages I and II) oocytes were homogenised in 500 μ l of Triton X-100 extraction buffer (0.5% Triton X-100, 10 mM Pipes [1,4-Piperazine-diethanesulphonic acid], pH 6.8, 0.3 M KCl, 10 mM MgOAc, 0.5 mM EGTA [ethylenebis oxyethylenitrilo tetraacetic acid], 20 μ g/ml yeast tRNA, 10 U/ml RNasin, 0.1 mM PMSF) using a Gilson p1000 Pipetman, and incubated at room temperature for 5-10 min. The samples were then centrifuged at 13 000 rpm for 5 min, and the supernatant (soluble fraction) removed to a new tube. Cytoskeletal pellets were then washed by adding 500 μ l of Triton X-100 extraction buffer and re-centrifuging for 5 min.

i. Total RNA extraction

Cytoskeletal pellets were resuspended in 400 μ l Extraction Solution A (50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 10 mM EDTA, 0.5% SDS, 25 μ g/ml yeast tRNA, 400 μ g/ml proteinase K), and digested at 45°C for 1 hr. Soluble fractions were diluted 1:4 with Solution A and also digested for 1 hr at 45°C. The RNA was extracted twice with phenol/chloroform (1:1) and precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol. RNA pellets were dissolved in TBE containing 14% formaldehyde for denaturing agarose gel electrophoresis and northern analysis as described in section 2.10.

ii. Immunoprecipitation

For immunoprecipitation assays of cytoskeletal fractions, cytoskeletal pellets were resuspended in 500 μ l Ipp150, and soluble fractions were diluted with 1 ml (1:2) Ipp150. Fractions were then added to protein A-Sepharose bound with 10 μ l anti-TFIIIA antiserum as described in section 2.6.I. Immunoprecipitation assays and subsequent RNA extraction were performed as described in section 2.6.I, after which RNA pellets were resuspended in TBE containing 14% formaldehyde for northern analysis as described in section 2.10.

II. Enrichment of Microtubules

To enrich oocyte microtubules, oocytes were fractionated essentially as described in section I, with the addition of 300 mM sucrose to the Triton X-100 extraction buffer. RNA was extracted and analysed as described above.

III. Enrichment of Intermediate Filaments

To enrich oocyte intermediate filaments, oocytes were fractionated using the procedure of Pondel and King (1988). Oocytes were homogenised in 500 µl of buffer containing 1% Triton X-100, 10 mM Pipes, pH 6.8, either 0.5 or 1.5 M KCl, 5 mM MgOAc, 1 mM EGTA, 300 mM sucrose, 20 µg/ml yeast tRNA, 10 U/ml RNasin, and 0.1 mM PMSF. Samples were incubated for 10 min at room temperature and centrifuged at 13 000 rpm for 5 min. RNA from each fraction was analysed as described in section I.

IV. Enrichment of Microfilaments

To enrich oocyte microfilaments, oocytes were fractionated using a low salt extraction buffer following a modified procedure from Capco and Bement (1991). Five hundred microlitres of low salt extraction buffer (92.5 mM KCl, 6.2 mM NaCl, 5 mM EGTA, 2 mM MgCl₂, 12 mM HEPES, pH 7.4, 1% Triton X-100, 20 mg/ml yeast tRNA, 10 U/ml RNasin, 0.1 mM PMSF) was added to oocytes in a microfuge tube, and incubated with rotation for 15 min at room temperature. The oocytes were then homogenised in the same buffer, and incubated a further 10 min at room temperature with rotation. Lysates were centrifuged for 5 min at 13 000 rpm, and pellet and supernatant fractions recovered. The RNA from the two fractions was analysed as described in section I.

2.12 ISOLATION OF INTRACELLULAR MEMBRANES

Oocyte membrane fractions were prepared as described in Colman (1984). Twenty mature (stage V) or immature (stages I and II) oocytes were homogenised in 500 µl ice-cold homogenisation buffer (0.15 M NaCl, 10 mM MgOAc, 20 mM Tris-HCl, pH 7.6, 10% sucrose, 10 U/ml RNasin, 1 mM PMSF) using a Gilson p1000 Pipetman, layered onto 1 ml of sucrose buffer (20% sucrose, 50 mM NaCl, 10 mM MgOAc, 20 mM Tris-HCl, pH 7.6, 10 U/ml RNasin, 1 mM PMSF) and centrifuged in a Sorvall SS-34 rotor at 11 500 rpm (15 000 x g) at 4°C for 30 min. After centrifugation the uppermost 500 µl (10% sucrose layer containing cytosolic components) was removed to a new tube. Pellets (containing membranes, yolk and pigment) were resuspended in 50 µl of homogenisation buffer and centrifuged for 1 min in a bench top microfuge at 10 000 rpm to pellet the yolk and pigment. Supernatants (containing membranes) were removed to new tubes.

I. Total RNA Extraction

RNA was extracted from membrane and cytosol fractions using the procedure of Peppel and Baglioni (1990). Membrane fractions were homogenised in 500 µl Solution 1 (2% SDS, 200 mM Tris-HCl, pH 7.5, 1 mM EDTA). Three cytosol fractions, of 166 µl each, were aliquoted into tubes and diluted with 334 µl (1:2) Solution 1. Each tube was then treated as described in section 2.8.I.i for extraction of RNA. After resuspension in TBE with 14% formaldehyde, cytosol fractions were recombined for gel electrophoresis and northern analysis.

II. Immunoprecipitation

For immunoprecipitation with anti-TFIIIA antiserum, membrane fractions were diluted with 450 µl Ipp150, and cytosol fractions were diluted with 500 µl Ipp150, before their addition to protein A-Sepharose bound to anti-TFIIIA antiserum.

Assays were subsequently performed as described in section 2.6, after which RNA pellets were resuspended in TBE with 14% formaldehyde for northern analysis.

CHAPTER 3

SEQUENCE AND STRUCTURAL REQUIREMENTS OF 5S RNA FOR PROTEIN ASSOCIATIONS AND RIBOSOME ASSEMBLY IN *XENOPUS* OOCYTES

3.1 INTRODUCTION

In living cells, RNA is usually complexed with proteins, to form ribonucleoprotein particles (RNPs). RNPs range in size from complexes of one RNA and one protein (for example, 7S RNPs), to large multicomponent complexes such as spliceosomes and ribosomes. The features of RNA that are involved in RNA-protein interactions are just beginning to be elucidated. Because RNA has so many diverse functions, but only consists of chains of four basic nucleotide subunits, a range of conformational flexibility is required, perhaps more analogous to that of protein structure than DNA structure. This versatility is achieved by the formation of RNA secondary and tertiary structures.

Secondary structures in RNA are formed by complementary regions within the molecule which form Watson-Crick base pairs. These base paired regions form right handed A-form double helices, which differ from the canonical B-form DNA helices in the distance of a complete helical turn and in the angle of the tilt that the

base pairs make with the helical axis (reviewed in Wyatt *et al.*, 1989). The consequence of these differences is manifested in the size and shape of the major and minor grooves. In B-form DNA the major groove is wide enough to accommodate protein structures such as an α -helix or an antiparallel β -ribbon, and the functional groups on the exposed edges of the base pairs can be directly contacted by side chains of proteins (reviewed in Steitz, 1993). The minor groove is deep and narrow, and so is less accessible to secondary structures such as α -helices; however, it is the major groove that contains a richer array of hydrogen bond acceptors and donors. A-form RNA helices contain a wide, shallow minor groove, and a major groove that is deep and narrow. The hydrogen bond acceptors and donors contained within the major groove are therefore largely inaccessible for protein interactions (reviewed in Steitz, 1993). However, most naturally occurring RNA molecules contain only relatively short duplex regions, which are interrupted by mismatches, bulges or loops. These features are thought to provide either sequence-specific contacts and/or conformational structures recognised by proteins (reviewed in Draper, 1989; Wyatt *et al.*, 1989; Wyatt and Tinoco, 1993; Draper, 1995)

As outlined in the General Introduction, 5S RNA associates with a number of proteins on its journey from synthesis to assembly in the ribosome in *Xenopus laevis* oocytes. In this chapter, I investigate the sequence and structural requirements of 5S RNA for binding to TFIIA and ribosomal protein L5, and for assembly into the 60S ribosomal subunit. Results from this study have been published (Allison *et al.*, 1993). A copy of this publication is included in Appendix III.

I. Secondary and Tertiary Structure of 5S RNA

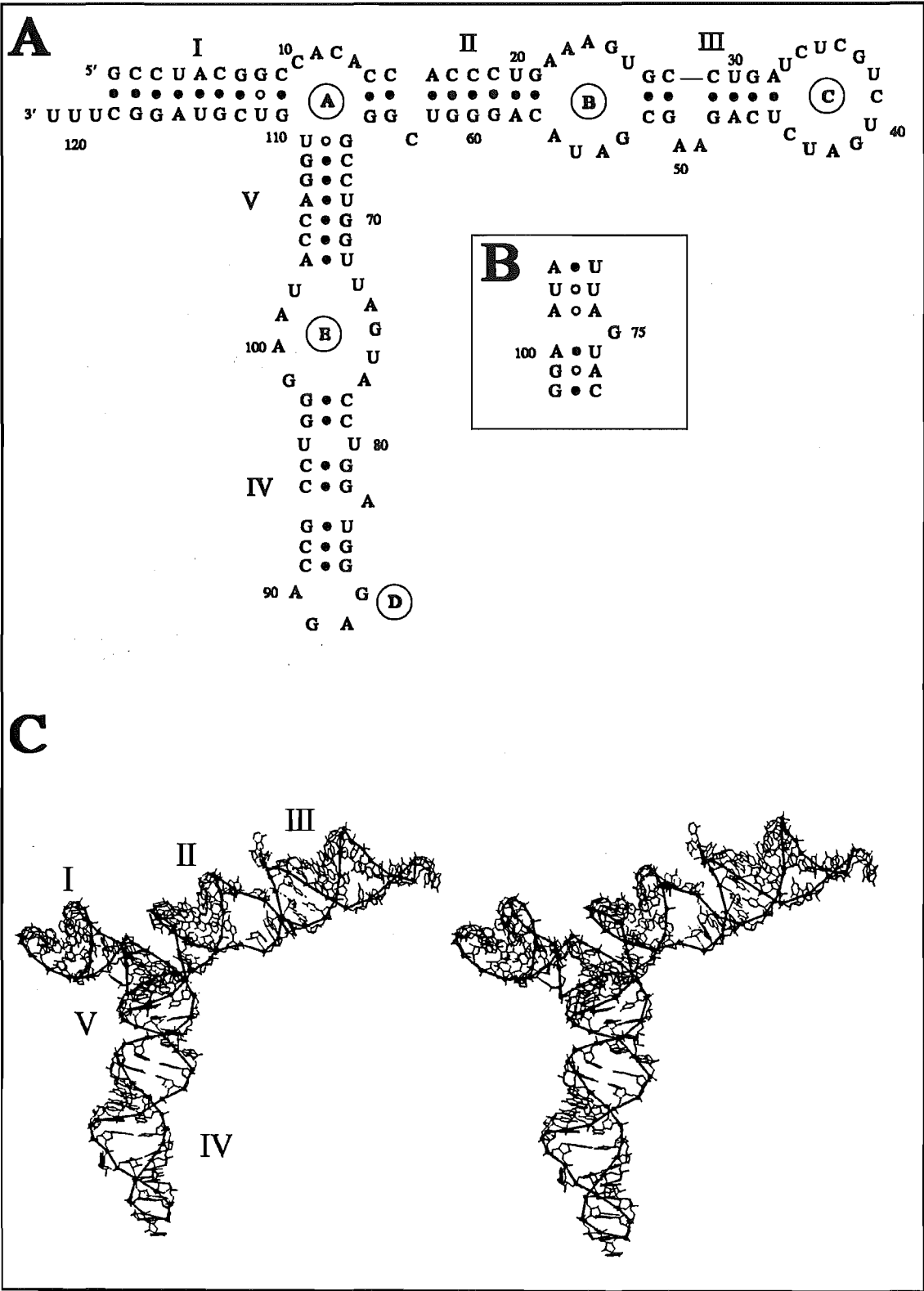
5S RNA is a small molecule of 120 nucleotides which shows sequence conservation throughout evolution (for compilation of sequences, see Specht *et al.*, 1990). Universal secondary structures have been proposed for both prokaryotic and eukaryotic 5S RNAs (reviewed in Garrett *et al.*, 1981). As shown in Figure 3-1A, the secondary structure of eukaryotic 5S RNA consists of five regions of Watson-Crick base pairing, referred to as helices I to V, which are connected by five single

stranded loops, designated A to E. Nucleotides are numbered from 1 to 120, in the direction 5' to 3'.

Analysis of the general consensus model of eukaryotic 5S RNA has indicated that the most highly conserved regions occur within the single stranded loops (Romaniuk, 1989). The structure of the asymmetric loop E has been the subject of many studies. Chemical and enzymatic probing, and nuclear magnetic resonance spectroscopy have suggested that loop E folds into an unusual secondary structure, containing non-canonical (non-Watson-Crick) base pairing, of the types A·A, U·U and A·G (Andersen *et al.*, 1984a; Romaniuk *et al.*, 1988; Wimberly *et al.*, 1993). The model for *Xenopus* loop E proposed by Wimberly *et al.* (1993) is shown in Figure 3-1B. Other features of the secondary structure of 5S RNA include four nucleotides which are bulged out of helical regions, which in *Xenopus* 5S RNA are at positions 49, 50, 63 and 83.

A model for the tertiary structure of *Xenopus* 5S RNA has been proposed, using computer graphic modelling (Westhof *et al.*, 1989). In this model, shown in Figure 3-1C, the 5S RNA molecule adopts a distorted Y-shape structure, the stalk of which consists of helix I and the two arms consisting of helices II and III and IV and V. Helices II and V are proposed to be almost colinear, and there are thought to be no long range tertiary interactions between loop C and loops D or E (Westhof *et al.*, 1989). Although other tertiary structure models have been proposed for 5S RNA which do incorporate long range tertiary interactions (for example, Pieler and Erdmann, 1982; Nazar, 1991), the above model has some experimental support. Mutagenesis studies showed that mutations in either loops B, C, D or E induced conformational changes restricted only to the mutated regions (Brunel *et al.*, 1990; Leal de Stevenson *et al.*, 1991). In addition, the tertiary structure of 5S RNA has been probed with bis (phen-anthroline)(phenanthrenequinone diimine) rhodium (III) ($\text{Rh}[\text{phen}]_2[\text{phi}]^{3+}$), which cleaves RNA preferentially at sites of tertiary interaction that provide an accessible major groove. The results of cleavage of wild-type 5S RNA as well as of mutated and truncated 5S RNA molecules indicate that the domains of 5S RNA are independently organised, and support the Y-model structure (Chow *et al.*, 1992).

Figure 3-1 Secondary and tertiary structure of *Xenopus laevis* oocyte-type 5S RNA. (A) Secondary structure of oocyte-type 5S RNA after Romaniuk *et al.* (1988). Double helix regions are numbered I to V, and single stranded loop regions are designated A to E. Loop E is depicted as single stranded. (B) Structure of loop E as proposed by Wimberly *et al.* (1993). Non-Watson-Crick base pairs are indicated by open circles. (C) Stereoscopic view of the three-dimensional model for the tertiary structure of *Xenopus* 5S RNA as proposed by Westhof *et al.* (1989), showing a Y-shape consisting of three independent domains. Reprinted with permission from B. Ehresmann.



II. TFIIA Binding

There have been many studies on the characterisation of the 7S RNP complex from *Xenopus* oocytes (reviewed in Romby *et al.*, 1990). Enzyme and chemical nuclease probing have shown that TFIIA protects a substantial portion of 5S RNA from cleavage, including helix II/loop B and helix IV/loop E/helix V (Andersen *et al.*, 1984b; Romaniuk, 1985; Huber and Wool, 1986a; Christiansen *et al.*, 1987; Darsillo and Huber, 1991; McBryant *et al.*, 1995). Deletion mutagenesis of 5S RNA showed that nucleotides 11-108 provide the necessary sequence and conformational information for TFIIA binding (Romaniuk *et al.*, 1987a). In addition, a series of 5S RNA mutants has been characterised for their *in vitro* binding affinity to TFIIA (Baudin and Romaniuk, 1989; Romaniuk, 1989; You and Romaniuk, 1990; Baudin *et al.*, 1991). The affinity binding data of these mutants is shown in Appendix I. The main conclusion to be drawn from these studies was that TFIIA appears to recognise essentially secondary structures of 5S RNA, predominantly the helical structure formed by the near colinear stacking of helices II and V.

Despite extensive studies *in vitro*, there is very little information regarding requirements for 7S RNP formation *in vivo*. I was therefore interested in determining the requirements of 5S RNA for binding TFIIA within the oocyte, and to compare this with the previously accumulated *in vitro* binding data. To this end, the series of mutant 5S RNA molecules previously analysed for their *in vitro* binding affinity to TFIIA was tested for their *in vivo* binding ability to TFIIA after microinjection into the oocyte cytoplasm. TFIIA binding was assessed by immunoprecipitation with an anti-TFIIA antibody, and by non-denaturing gel electrophoresis.

III. L5 Binding

Since binding of 5S RNA to ribosomal protein L5 (YL3 or L1 in yeast; L5, L18 and L25 in *E. coli*) seems to be a precursor to assembly into the large ribosomal subunit in bacteria, yeast, amphibians and mammals (Yu and Wittmann, 1973; Steitz

et al., 1988; Allison *et al.*, 1991; Deshmukh *et al.*, 1993), then an obvious prerequisite for ribosome incorporation of 5S RNA is binding to L5.

The 5S RNA-binding protein(s)-5S RNA complex has been most extensively studied in *E. coli*. Nuclease protection assays showed that the three *E. coli* 5S RNA-binding proteins each bind a different site on 5S RNA, with L18 having the highest affinity and aiding the subsequent binding of L5 (Pieler and Erdmann, 1982; Huber and Wool, 1984; Egebjerg *et al.*, 1989). More detailed studies of L18 binding revealed the importance of a bulged adenosine residue at position 66 in helix II (Peattie *et al.*, 1981; Christiansen *et al.*, 1985; Egebjerg *et al.*, 1989). The position of this bulged nucleotide is invariant within helix II in 5S RNA from different species, although its identity varies with major phylogenetic divisions (Peattie *et al.*, 1981).

Binding studies of yeast ribosomal protein L1 to 5S RNA have shown that helices I, II, and IV are important for this interaction (Nazar, 1979; Nazar and Wildeman, 1983). In rat liver, helices II, IV and V of 5S RNA were protected by L5 binding from the cytotoxic nuclease, α -sarcin (Huber and Wool, 1986b). These studies reveal that the site of ribosomal protein binding on 5S RNA is conserved in different organisms, and that the binding site of rat L5 and yeast L1 approximates that of the combined binding sites of the three *E. coli* 5S RNA-binding proteins (Nazar and Wildeman, 1983; Huber and Wool, 1986b; reviewed in Nazar *et al.*, 1982).

The mutant 5S RNAs used in the present study have also been previously analysed for their *in vitro* binding affinity to *Xenopus* L5 (Q. You, W. Q. Zang, and P. J. Romaniuk, in prep.). This data is shown in Appendix I. Surprisingly, most of the 5S RNA mutants tested were not significantly affected in their binding to L5, including deletion of the bulged cytidine residue in helix II. Mutations which altered the binding affinity of L5 the most were located in helix III. Slightly differing results were recently obtained by another group, also investigating the binding of *Xenopus* 5S RNA to L5 (Scripture and Huber, 1995). In this study, 5S RNA mutants were identified which significantly altered the binding affinity of L5. These results were similar to the above results in that the mutations most defective for L5 binding were confined to the helix III/loop C region of 5S RNA. The reason for the discrepancy between the two sets of data is not known, but may be due to the different assays used by each group to determine L5 binding. In order to test the ability of the

5S RNA mutants to bind L5 *in vivo*, I performed non-denaturing gel electrophoresis on oocyte homogenates after microinjection of mutant 5S RNAs into the oocyte cytoplasm.

IV. 5S RNA within the Ribosome: Structure and Function

Ribosomal RNA is thought to perform more than just a structural role within the ribosome. Evidence indicates that rRNA may have a role in all steps of protein synthesis, including messenger RNA (mRNA) binding, subunit association, transfer RNA (tRNA) binding and peptidyltransferase activity (reviewed in Dahlberg, 1989; Noller, 1993). The function of 5S RNA is still unknown, but its universal presence and high degree of conservation suggest it has an important role. Prokaryotic ribosomes reconstituted *in vitro* lacking 5S RNA show greatly reduced activities, including peptidyltransferase, tRNA binding, elongation factor-dependent binding of GTP and chain termination (Dohme and Nierhaus, 1976; Erdmann, 1976).

Complexes formed *in vitro* between 5S RNA and 18S rRNA have led to the hypothesis that this interaction may be important for the association of the 60S subunit with the 40S subunit (Azad and Lane, 1973). Subsequent analyses have delineated the binding sites of this interaction to be at the 3' end of both RNAs in *Neurospora* (Kelly and Cox, 1982), and to a 5' region and a 3' region in mouse 5S RNA (Sarge and Maxwell, 1991). Another proposed function of 5S RNA in the ribosome is in binding to aminoacyl-tRNAs via base pairing of the conserved tRNA GT Ψ C sequence with the conserved GAAC sequence (loop C) of bacterial 5S RNA (Erdmann, 1976). However, 50S ribosomal subunits containing 5S RNA with deletions of this and surrounding sequences retained their ability to carry out poly(A)-directed synthesis of polyphenylalanine and to translate a natural phage mRNA *in vitro*, ruling out this interaction being an obligatory step for protein synthesis (Pace *et al.*, 1982; Zagorska *et al.*, 1984). Yet another postulated function of 5S RNA is as a binding site for eukaryotic elongation factor 2 and associated GTPase activity (Grummt *et al.*, 1974; Nygård and Nilsson, 1987; Holmberg *et al.*, 1992).

Possible functions of 5S RNA can also be surmised from its topographical position within the ribosome. The morphologies of prokaryotic and eukaryotic ribosomes have been established by three-dimensional electron microscopy (reviewed in Frank *et al.*, 1990). Ribosomes from the two kingdoms show a marked degree of morphological resemblance. Common features of the large ribosomal subunit include the central protuberance, the interface canyon, and stalk and ridge structures (Fig. 3-2). The peptidyltransferase centre is thought to lie in the vicinity of the interface canyon, at the base of the central protuberance (reviewed in Frank *et al.*, 1990). To date, most analyses of ribosomal structure have used prokaryotic species. The structures of the *E. coli* 30S and 50S ribosomal subunits have been fairly well mapped with regard to protein placement using immunoelectron microscopy and protein-protein crosslinking (Walleczek *et al.*, 1988; reviewed in Brimacombe *et al.*, 1990; Oakes *et al.*, 1990; Stöffler-Meilicke and Stöffler, 1990).

The three *E. coli* 5S RNA-binding proteins, L5, L18 and L25, have been localised near the top of the central protuberance of the 50S subunit (Fig. 3-2; Walleczek *et al.*, 1988). Immunoelectron microscopy has placed the 3'-5' terminal stem of 5S RNA on the surface of the 50S subunit, on the outward side of the central protuberance (Shatsky *et al.*, 1980; Stöffler-Meilicke *et al.*, 1981; Clark and Lake, 1984), whereas loop C was mapped to the interface side of the central protuberance (Evstafieva *et al.*, 1985). In addition, crosslinking studies have suggested that loop D lies close to the peptidyltransferase centre, and a model was proposed such that 5S RNA is constrained into a bent Y shape within the *E. coli* ribosome (Fig. 3-2; Dontsova *et al.*, 1994).

The placement of ribosomal proteins within the eukaryotic 60S subunit has not been established. However, crosslinking studies on rat liver 80S ribosomes showed protein L5 crosslinked with two 40S subunit proteins, S4 and S25, indicating L5 lies somewhere near the subunit interface (Uchiumi *et al.*, 1986). In addition, L5 was also identified as being located at the peptidyl-tRNA binding site (Fabijanski and Pellegrini, 1981).

Although the above data are by no means definitive, it appears that in both prokaryotic and eukaryotic ribosomes, 5S RNA and binding protein(s) occupy a similar and functionally significant position. There is, however, very little information concerning the actual sequence and/or conformational structures of

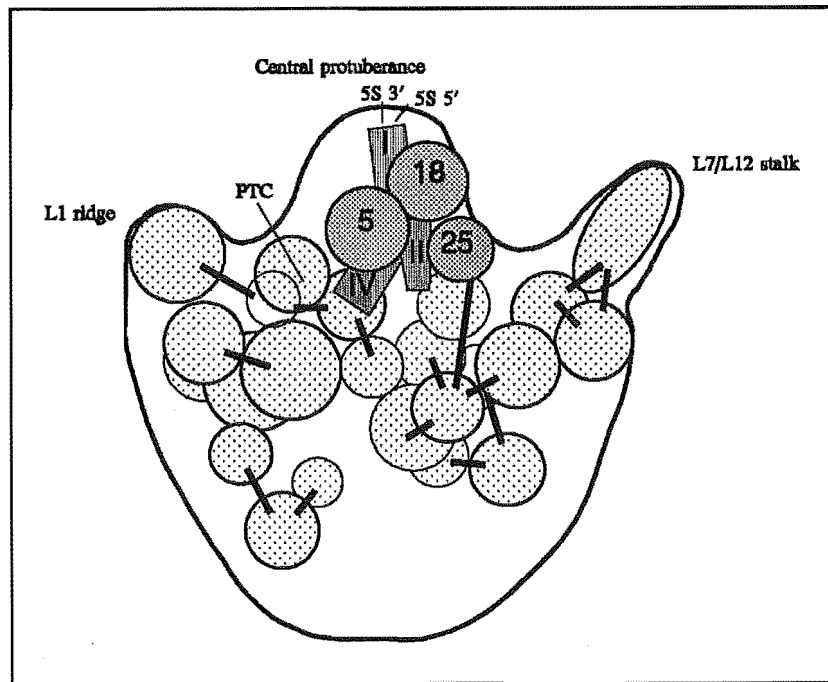


Figure 3-2 Diagram of prokaryotic 50S large ribosomal subunit, showing proposed location of 5S RNA and binding proteins. The figure shows the "crown projection" of the large subunit, with the interface side facing the viewer. PTC, peptidyltransferase centre; 5S 5' and 5S 3', 5' and 3' termini of 5S RNA. Location of proteins (indicated by shaded circles and numbers) and protein-protein crosslinks are from Walieczech *et al.* (1988). The location of the three arms of 5S RNA (I, helix I; II, helices II and III; IV, helices IV and V) are derived from data from Shatsky *et al.* (1980), Stöffler-Meilicke *et al.* (1981), Clark and Lake (1984), Evstafieva *et al.* (1985) and Dontsova *et al.* (1994).

5S RNA that are required for integration into ribosomal subunits. To determine these requirements in *Xenopus*, I have analysed the ability of a series of mutant 5S RNA molecules to be incorporated into 60S ribosomal subunits after microinjection into the oocyte cytoplasm. This was assayed by immunoprecipitation using an anti-60S ribosomal subunit antibody.

V. Description of 5S RNA Mutants used in this Study

A summary of the 5S RNA mutants used in this study is shown in Figure 3-3. To test the importance of the highly conserved single stranded loops of 5S RNA, a series of mutant molecules were used which contained block substitutions in each of the loop regions. These substitutions maintain the single stranded secondary structure,

but with a different nucleotide sequence (Romaniuk, 1989). Mutants are designated by the nucleotides which have been substituted, for example, in mutant 10-13, nucleotides at positions 10 to 13 (CACA) have been substituted with the sequence UGCG. Another loop mutation, 96-101, has three nucleotide substitutions in the loop E region designed to increase the amount of Watson-Crick base pairs, to test the importance of the unusual conformation in this region of the molecule (Romaniuk, 1989).

A second group of mutant 5S RNA molecules contains mutations in the helical stem regions. These mutants also consist of block substitutions, on either side of a double helix, resulting in mismatches and the formation of single stranded regions. Double mutants contain compensating substitutions on both sides of the helix, thus restoring the helical stem structure, but with a different nucleotide sequence (You and Romaniuk, 1990). This allowed the importance of both the helical structure and sequence to be tested for protein interactions.

To test the importance of the bulged nucleotides for protein associations, mutants were tested in which the bulged nucleotides at position 49 and 50, 63 or 83 were deleted (Baudin and Romaniuk, 1989). These mutants are referred to as $\Delta 49,50$, $\Delta 63$ and $\Delta 83$, respectively.

Nucleotides at the junction of helices I, II and V are highly conserved, and have been proposed to control the colinearity of helices II and V (Romaniuk, 1989; Baudin *et al.*, 1991). Two further 5S RNA mutants were therefore tested, in which nucleotides G66 and U109 were replaced with a cytidine and guanosine, respectively (Baudin *et al.*, 1991).

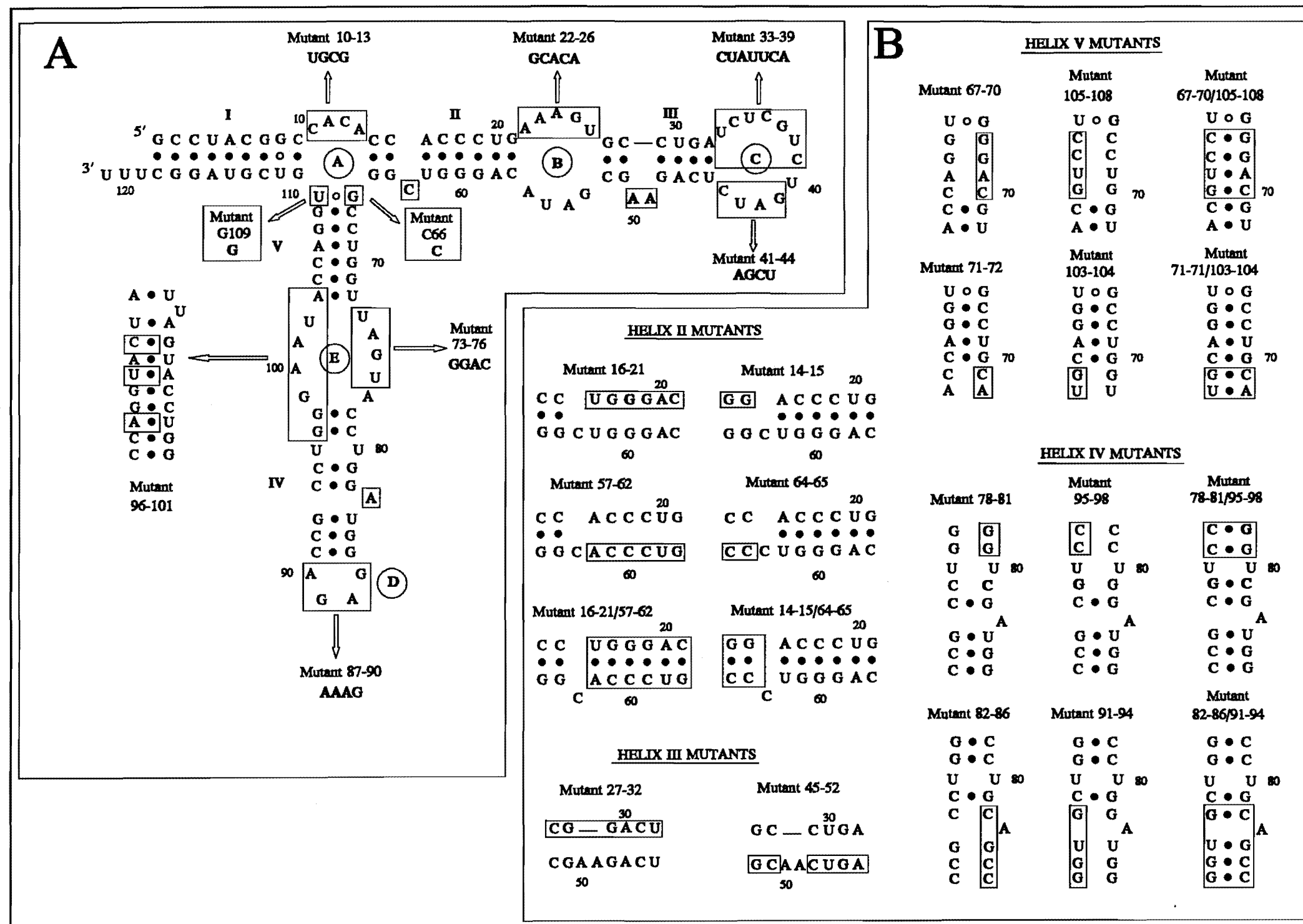


Figure 3-3 Secondary structure of *Xenopus laevis* oocyte-type 5S RNA showing mutant nucleotide substitutions and deletions. (A) Single strand substitutions. Boxed nucleotides were replaced with the indicated nucleotides. The bulged nucleotides deleted at positions 49, 50, 63 and 83 are indicated in boxes. (B) Helix mutants. Only the relevant region of 5S RNA is shown; substituted nucleotides are indicated in boxes.

3.2 RESULTS

To determine the sequence and structural requirements of 5S RNA for fulfilling its various protein associations *in vivo*, mutant ^{32}P -labelled 5S RNAs were synthesised using T7 RNA polymerase-mediated *in vitro* transcription. The mutant 5S RNAs were microinjected into the cytoplasm of stage V-VI oocytes and incubated 48 hours before analysis by immunoprecipitation or non-denaturing gel electrophoresis. Antisera for TFIIA and 60S ribosomal subunits were kindly provided by Dr M. le Maire (CEA et CNRS, Gif-sur-Yvette, France).

I. Characterisation of Antisera

The anti-TFIIA antiserum reacts specifically with TFIIA; no cross-reaction was noted with any other protein by immunoblotting (Viel *et al.*, 1990; Allison *et al.*, 1993). The anti-TFIIA antiserum also recognises 7S RNPs: immunoprecipitation with the anti-TFIIA antiserum of stage V oocytes labelled *in vivo* with [^{32}P]GTP revealed coprecipitation of 5S RNA only (Fig. 3-4A, lane 1), whereas normal rabbit serum did not precipitate any RNA species (lane 5). The coprecipitability of 5S RNA was destroyed by prior treatment of oocyte extracts with proteinase K (lane 2), showing the antibody is specific for the protein component of 7S RNPs and does not recognise the naked 5S RNA molecule.

The anti-60S ribosomal subunit antiserum detects two major bands in extracts of 60S ribosomal subunits: a 37 kD band corresponding to ribosomal protein L2; and a 14-15 kD protein which migrates similarly to ribosomal proteins in the range L17-L20 (Allison *et al.*, 1993). This antiserum does not immunoprecipitate 7S RNPs (Allison *et al.*, 1993), but does precipitate intact 80S ribosomes, as demonstrated by the presence of the 40S subunit RNA, 18S rRNA, in immunoprecipitates (data not shown), and the presence of proteins that react with anti-40S ribosomal subunit antiserum (Allison *et al.*, 1993).

In vivo labelling of oocytes with [^{32}P]GTP overnight, followed by immunoprecipitation with the anti-60S subunit antiserum did not precipitate

³²P-labelled 5S RNA, although a higher molecular weight RNA, which is probably 5.8S rRNA, was precipitated (Fig. 3-4A, lane 3). This RNA was not precipitated after proteinase K digestion (lane 4) or with normal rabbit serum (lane 5), indicating it is specifically precipitated with the anti-60S antiserum. The lack of 5S RNA in immunoprecipitates probably reflects the small amount of 5S RNA synthesised in stage V oocytes (as shown in immunosupernatant fractions in Figure 3-4B), and also the presence of a pool of unlabelled 5S RNA in the nucleus (see Chapter 4). Similar results were noted by Knight and Darnell (1967) in HeLa cells.

II. Analysis of Mutant RNAs for Incorporation into 7S RNPS and 60S Ribosomal Subunits by Immunoprecipitation

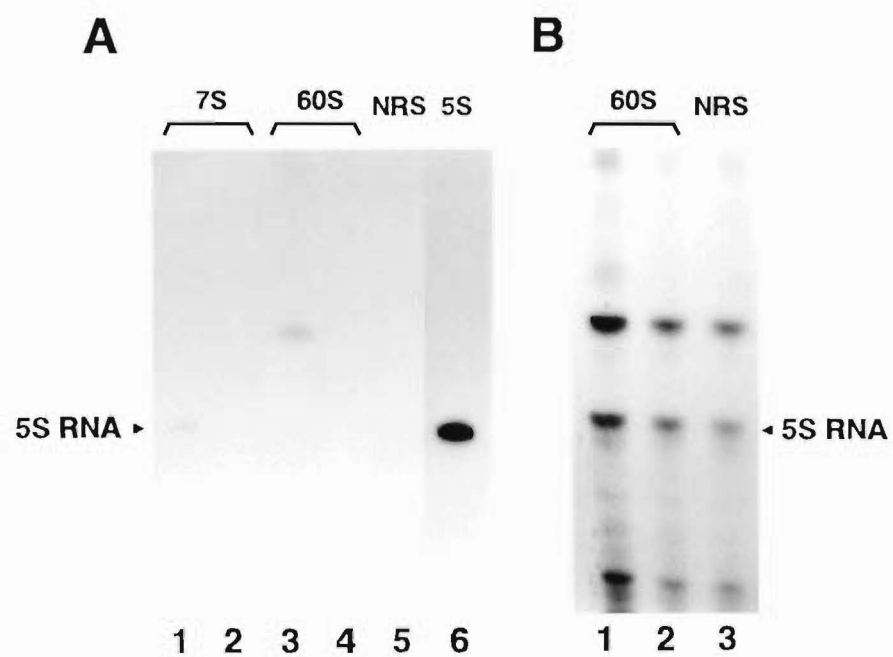
i. 7S RNP formation

To assess the formation of 7S RNPs after injection of mutant 5S RNAs into the cytoplasm of oocytes, oocyte homogenates were immunoprecipitated with the anti-TFIIIA antiserum. The RNA was then extracted from the immunoprecipitates and analysed by polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Figure 3-5 shows the results of immunoprecipitation of a representative selection of 5S RNA mutants, and the complete results are presented in Table 3-1. Each mutant 5S RNA was assayed in a minimum of two different batches of oocytes. Since these assays are not quantitative owing to the large amounts of endogenous 5S RNA and RNPs, RNP formation is expressed as either positive or negative in Table 3-1. Immunoprecipitates (Fig. 3-5A) of mutant 5S RNAs were compared with that of oocyte-type 5S RNA within each assay, as well as with immunosupernatant fractions (Fig. 3-5B). Immunosupernatants confirmed that approximately equal amounts of each RNA were injected per oocyte, and showed that the injected RNA was stable.

The majority of mutant 5S RNA molecules tested showed similar 7S RNP formation to oocyte-type (lane 1), for example, mutants 78-81 (lane 3) and $\Delta 49,50$, $\Delta 63$ and $\Delta 83$ (lanes 15, 17 and 19, respectively). Out of the 32 mutant 5S RNAs

Figure 3-4 Immunoprecipitation of endogenous oocyte RNAs with anti-TFIIIA and anti-60S subunit antisera. Oocyte RNAs were labelled by injection of [32 P]GTP into the cytoplasm of stage V oocytes followed by an overnight incubation. (A) Immunoprecipitations were performed with anti-TFIIIA (7S), anti-60S ribosomal subunit (60S) antisera or with normal rabbit serum (NRS). Samples shown in lanes 2 and 4 were subjected to digestion with proteinase K before immunoprecipitation. RNA was extracted from immunoprecipitates and analysed by 8 M urea/8% PAGE and autoradiography. Lane 6, a sample of 32 P-labelled 5S RNA as a marker. (B) Immunosupernatant fractions were recovered from reactions in lanes 3 to 5 of panel A. Immunoprecipitates were exposed to X-ray film for four weeks, and immunosupernatants for three weeks.



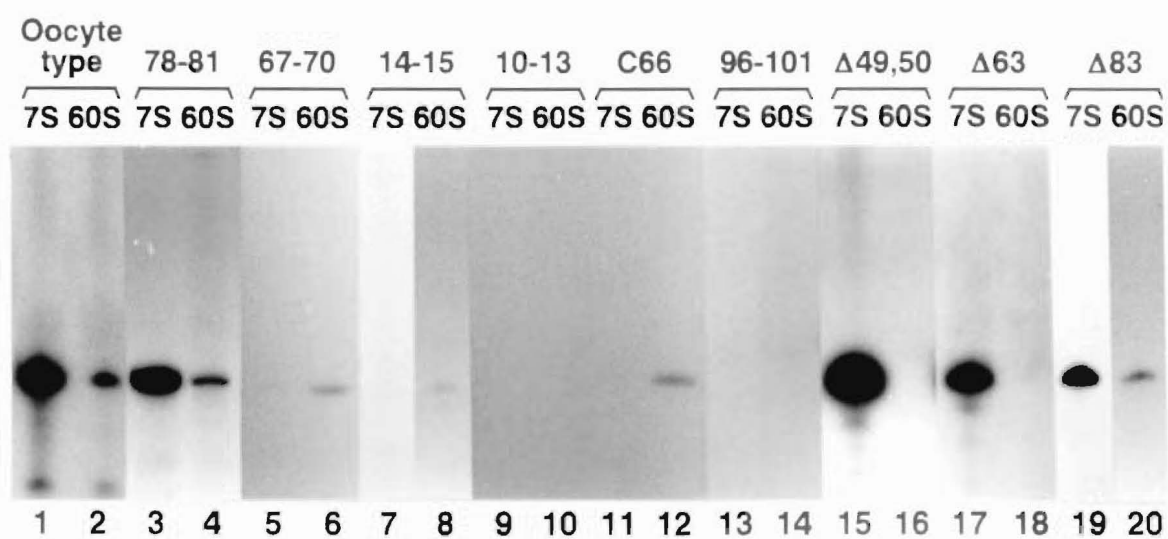
tested, eight were not immunoprecipitated with the anti-TFIIIA antiserum. Three of these mutants, 10-13 (lane 9), C66 (lane 11) and G109 (not shown), with mutations centred around the loop A region of 5S RNA, agree with the *in vitro* binding data previously established for these mutants (K_a 0.30, 0.12 and 0.17 relative to oocyte-type; Romaniuk, 1989; Baudin *et al.*, 1991). However, many of the mutants found defective for TFIIIA binding by immunoprecipitation, for example mutants 14-15 (lane 7), 64-65 (not shown) and 67-70 (lane 5), do not show significantly impaired binding affinities for TFIIIA *in vitro* (see Appendix I; You and Romaniuk, 1990). Thus, to confirm the results of the immunoprecipitation assays, 7S RNP formation was also assayed by non-denaturing PAGE, as described in section III.

ii. 60S ribosomal subunit incorporation

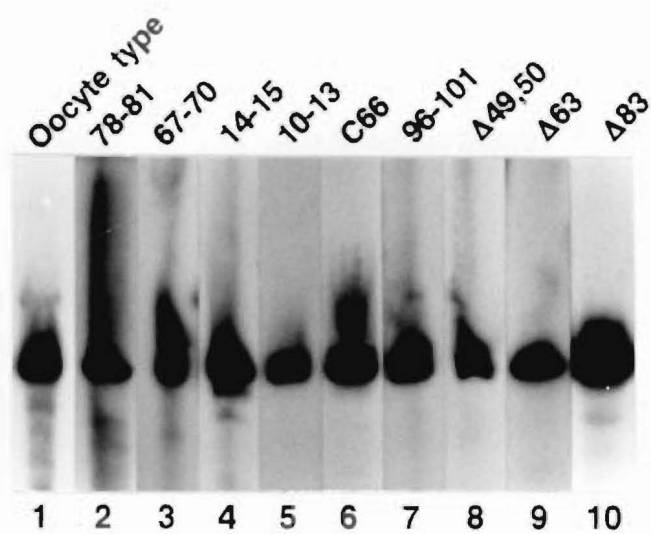
To test the ability of the mutant 5S RNAs to be incorporated into 60S ribosomal subunits after microinjection into the cytoplasm of oocytes, oocyte homogenates were immunoprecipitated with the anti-60S subunit antiserum. Figure 3-5 shows the results of a representative set of mutant 5S RNA molecules after immunoprecipitation with anti-60S subunit antiserum. The complete results are presented in Table 3-1. As with the anti-TFIIIA immunoprecipitations, these immunoprecipitations are qualitative only, because of the huge amounts of ribosomes which have accumulated by late oogenesis. The results are therefore presented as either positive or negative. Most of the mutant 5S RNAs were precipitated with this antiserum, indicating stable assembly into 60S subunits, for example mutants 78-81 (lane 4), 67-70 (lane 6), 14-15 (lane 8), C66 (lane 12) and $\Delta 83$ (lane 20). Of the 32 mutants tested, only four were reproducibly defective for incorporation into the 60S subunit: mutant 10-13 (lane 10), which contains nucleotide substitutions in loop A; 96-101 (lane 14), where base substitutions have abolished the non-canonical base pairing in favour of Watson-Crick base pairs; and mutants $\Delta 49,50$ and $\Delta 63$ (lanes 16 and 18), in which bulged nucleotides have been deleted. These results indicate that these regions of the 5S RNA molecule are important for assembly into the 60S ribosomal subunit.

Figure 3-5 Assembly of mutant 5S RNAs into 7S RNPs and 60S ribosomal subunits assayed by immunoprecipitation. ³²P-labelled mutant 5S RNAs were microinjected into the oocyte cytoplasm. After 48 hr, homogenates of whole oocytes were immunoprecipitated with anti-TFIIIA (7S) or anti-60S ribosomal subunit (60S) antisera. Labelled RNAs were recovered from immunoprecipitates (A) and immunosupernatants (B) and analysed by 8 M urea/8% PAGE and autoradiography. Immunoprecipitates were exposed to X-ray film for 3 weeks and immunosupernatants overnight. Numbers refer to those nucleotides which have been substituted or deleted (Fig. 3-3).

A



B



III. Analysis of Mutant RNAs for 7S RNP and 5S RNP Formation by Non-denaturing PAGE

i. 7S RNP formation

Since some of the 5S RNA mutants not immunoprecipitated with the anti-TFIIIA antiserum showed near wild-type *in vitro* affinities for TFIIIA, formation of 7S RNPs was also analysed by non-denaturing gel electrophoresis. Analysis of oocyte homogenates after injection of ^{32}P -labelled 5S RNA reveals a band of free 5S RNA and two 5S RNA-containing species of slower mobility (Fig. 3-6). The identity of the slower of these two bands as corresponding to 7S RNPs was established by simultaneously running a sample of unlabelled purified 7S RNPs and staining with ethidium bromide (Allison *et al.*, 1993).

A selection of mutant 5S RNAs analysed by this method is shown in Figure 3-6, and the complete results are summarised in Table 3-1. Only one mutant, 10-13, was shown to be defective for TFIIIA binding using this assay (lanes 3 and 4). The other seven 5S RNA mutants which were not immunoprecipitated with the anti-TFIIIA antibody (Fig. 3-5) all show bands corresponding to 7S RNPs (lanes 5 to 11), although there is variation in the density of the bands. These conflicting results imply that 7S RNP complexes containing these mutant 5S RNAs were not recognised by the anti-TFIIIA antiserum, suggesting that they have an altered conformation. This is supported by closer examination of the mutant molecules in this category. Mutants C66 and G109 contain substitutions of residues at the junction of helices I, II and IV, a region known to be important for the maintenance of the tertiary structure of 5S RNA (Baudin *et al.*, 1991). Also, mutants 73-76 and 96-101 contain modifications in the loop E region of the molecule, which is known to form a complex tertiary structure consisting of non-Watson-Crick base pairing (Westhof *et al.*, 1989; Wimberly *et al.*, 1993). The remaining three mutants, 64-65, 67-70 and 14-15 all contain base substitutions in one side of either helix II or V, thus destroying the helical structure of the molecule. Thus, all of these mutations have the potential to significantly alter the conformation of the 5S RNA and consequently that of the

resulting 7S RNP. It is also possible that, although these mutants bind TFI_{II}A, the RNP is less stable under the conditions of immunoprecipitation. Alternatively, the positive results for these mutants revealed by non-denaturing electrophoresis could be due to the binding of another protein of similar size to TFI_{II}A, resulting in a complex with identical mobility to 7S RNPs. However, given that many of these mutant 5S RNAs showed *in vitro* binding affinities similar to wild type 5S RNA, and that there are no other known 5S RNA-binding proteins of this size in stage V oocytes, this explanation seems unlikely.

The occurrence of 'false negatives,' is not a concern with the anti-60S subunit antiserum. The ribosomal proteins recognised by this antiserum do not directly bind 5S RNA and have not been localised to the same region of the 60S subunit as 5S RNA. These proteins are therefore unlikely to be susceptible to any local conformational changes that may be induced in 60S subunits containing the mutant 5S RNA molecules.

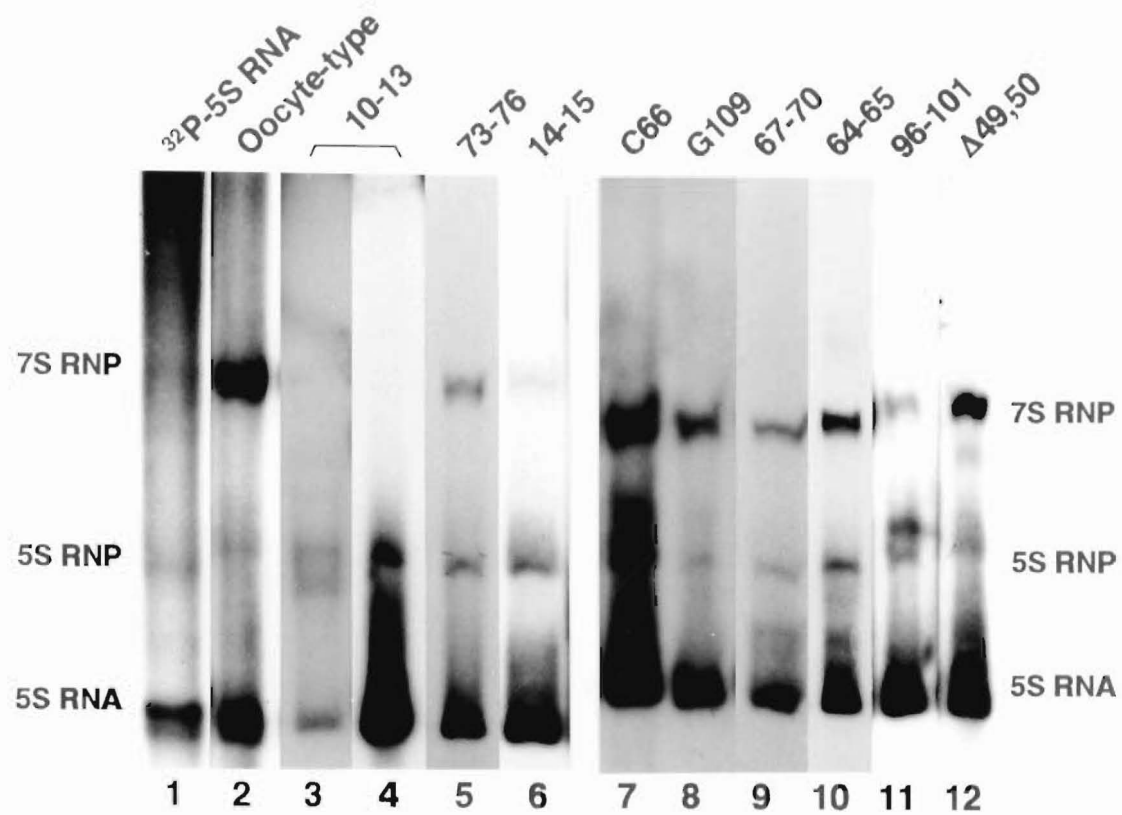
In summary, only one mutant, 10-13, consisting of base substitutions in loop A, out of the 32 mutant 5S RNA molecules tested *in vivo*, was completely defective for binding TFI_{II}A after microinjection into the oocyte cytoplasm.

ii. 5S RNP formation

The binding of ribosomal protein L5 to 5S RNA to form a 5S RNP is thought to be a precursor to ribosome assembly in a wide range of organisms (Yu and Wittmann, 1973; Steitz *et al.*, 1988; Allison *et al.*, 1991; Deshmukh *et al.*, 1993), and is also thought to play a role in targeting 5S RNA to the nucleus in *Xenopus* oocytes (Allison *et al.*, 1991; 1993; K. J. Murdoch and L. A. Allison, submitted). Analysis of the results of non-denaturing PAGE of homogenised oocytes, after cytoplasmic microinjection of ³²P-labelled 5S RNA, reveals an additional band of intermediate mobility between free 5S RNA and 7S RNPs (Fig. 3-6, lane 2). This band was shown to correspond to 5S RNA bound to L5 (Allison *et al.*, 1995). Thus, this assay also provided a means of testing the mutant 5S RNAs for their ability to bind L5 *in vivo*.

Examination of the oocyte-type 5S RNA-containing complexes after non-denaturing electrophoresis shows only a faint band corresponding to 5S RNPs: the

Figure 3-6 Non-denaturing gel electrophoresis of mutant 5S RNAs showing formation of 5S RNPs and 7S RNPs. ^{32}P -labelled mutant 5S RNAs were microinjected into the cytoplasm of oocytes. After incubation for 48 hr, oocyte homogenates were electrophoresed on 6% polyacrylamide gels containing 0.1% Triton X-100, followed by exposure to X-ray film for 1 to 14 days. Numbers above the lanes refer to nucleotides which were substituted or deleted (Fig. 3-3). Lane 1, sample of ^{32}P -labelled 5S RNA as a marker. Lanes 3 and 4 show the results for mutant 10-13 in two different batches of oocytes. Lanes 2 to 6 and 7 to 12 represent experiments in which samples were electrophoresed for different lengths of time.



majority of 5S RNA is either bound to TFIIIA or exists free (Fig. 3-6, lane 2). This pattern of RNP formation was highly reproducible within many different batches of oocytes, irrespective of the amount of 5S RNA injected. Analysis of the mutant 5S RNA molecules reveals different patterns. For example, mutants 10-13 and 14-15 (lanes 3, 4 and 6) show preferential binding to L5, with only low (14-15), or no (10-13) amounts of 7S RNP detectable. To take this variation into account, RNAs showing a strong preference for either L5 or TFIIIA have been recorded in Table 3-1 as "+++", whereas a single "+" merely denotes the presence of a particular RNP.

All of the mutant 5S RNAs tested formed detectable complexes with L5 (Fig. 3-6, lanes 2 to 12). These results concur with the *in vitro* binding data presented in Appendix I, where none of the mutants were significantly impaired for L5 binding (Q. You, W. Q. Zang, P. J. Romaniuk, in prep.).

Table 3-1 Summary of incorporation of mutant 5S RNAs into oocyte RNPs

Region of Molecule	5S RNA Mutant ^a	Immunoprecipitation ^b		Non-denaturing PAGE ^c	
		anti 7S	anti 60S	7S RNP	5S RNP
	Oocyte type	+	+	+++	+
<u>Helix II</u>	14-15	-	+	+	+
	64-65	-	+	+	+
	14-15/64-65	IR	+	+	+
	16-21	+	+	ND	ND
	57-62	+	+	+	+
	16-21/57-62	+	+	+	+
<u>Helix III</u>	27-32	+	+	+	+
	45-52	+	+	+	+
<u>Helix IV</u>	78-81	+	+	ND	ND
	95-98	+	+	+	+
	78-81/95-98	+	+	ND	ND
	82-86	+	+	ND	ND
	91-94	+	+	+	+
	82-86/91-94	+	+	ND	ND
<u>Helix V</u>	67-70	-	+	+	+
	105-108	+	+	ND	ND
	67-70/105-108	+	IR	+	+
	71-72	+	IR	ND	ND
	103-104	+	+	ND	ND
	71-72/103-104	+	+	ND	ND
<u>Loop A</u>	10-13	-	-	-	+
<u>Loop B</u>	22-26	+	+	+	+
<u>Loop C</u>	33-39	+	+	+	+
	41-44	+	+	+	+
<u>Loop D</u>	87-90	+	+	+	+
<u>Loop E</u>	73-76	-	+	+	+
	96-101	-	-	+	+
<u>Bulged nucleotides</u>	Δ49,50	+	-	+	+
	Δ63	+	-	+	+
	Δ83	+	+	+	+++
<u>Hinge nucleotides</u>	C66	-	+	+	+
	G109	-	+	+	+

^a The numbers in the designations refer to those 5S RNA nucleotides which have been substituted or deleted (Fig. 3-3).

^b Assays were performed as described in the legend to Figure 3-5. IR, inconclusive results due to RNA with low specific activity.

^c Assays were performed as described in the legend to Figure 3-6. A single "+" denotes the presence of a RNP, whereas "+++" indicates the RNA showed a strong preference for a particular RNP. ND, not determined.

3.3 DISCUSSION

The results described above further characterise regions of the 5S RNA molecule important for protein binding and ribosome assembly within *Xenopus* oocytes. Surprisingly, the majority of introduced modifications were tolerated for the various interactions, and all of the mutant RNAs were stable in oocytes, probably because all could bind to either TFIIA or L5.

I. TFIIA Binding

Of all the mutations tested, only one, with base substitutions at residues 10-13 in loop A, was completely defective in binding TFIIA, as judged by both immunoprecipitation and non-denaturing PAGE assays. This mutant has also been shown to have a low *in vitro* binding affinity to TFIIA (0.3 relative to oocyte-type; Romaniuk, 1989). These results suggest that loop A either provides base-specific contacts for interaction with TFIIA, or is important in maintaining the correct conformation of 5S RNA for TFIIA binding. The former is not supported by numerous footprinting experiments conducted on the 5S RNA-TFIIA complex, where protection of loop A was never observed (Andersen *et al.*, 1984b; Romaniuk, 1985; Huber and Wool, 1986a; Christiansen *et al.*, 1987). It has been proposed, therefore, that the residues of loop A may act to orient helices II and V optimally for the interaction with TFIIA (Romaniuk, 1989; Baudin *et al.*, 1991).

Some of the results presented here do not completely correlate with the *in vitro* binding data, highlighting the importance of the cellular context. Several mutants with low *in vitro* binding affinities to TFIIA (similar to that of 10-13), for example C66, G109 and 41-44 (see Appendix I), were clearly capable of binding TFIIA *in vivo*. These results reflect the situation in the oocyte, where TFIIA is not the only protein 5S RNA can bind to once injected into the oocyte cytoplasm, and where the mutants must compete with the endogenous, wild-type 5S RNA for protein binding. If mutants C66, G109 and 41-44 have an even lower affinity for another 5S RNA-binding protein, for example L5, then they may be more likely to bind

TFIIIA in the oocyte. It is interesting to note in this context that mutant 10-13 shows a high degree of L5 binding *in vivo*, whereas C66, G109 and 41-44 show moderately low levels of L5 binding.

Many of the mutant 5S RNAs tested here have also previously been tested for their ability to compete with wheat germ 5S RNA in binding TFIIIA (Baudin and Romaniuk, 1989; Romaniuk, 1989; Baudin *et al.*, 1991). These competition results are also presented in Appendix I. This assay is thought to provide a more sensitive indication of the specificity of the TFIIIA-5S RNA interaction (Romaniuk, 1985). Comparison of the *in vitro* competition strengths reveals that mutant 10-13 is approximately twenty-fold less effective in competing for TFIIIA binding than wild-type 5S RNA (Romaniuk, 1989), whereas mutants C66 and G109 show only a five-fold reduction and mutant 41-44 showed only a two-fold reduction in competition strength compared with wild-type (Baudin *et al.*, 1991). Thus, the differences observed *in vivo* between mutants 10-13 and C66, G109 and 41-44 possibly reflect this difference in their ability to compete with endogenous, wild type 5S RNA. The above results emphasise the complexity of the intracellular setting, but also highlight the value of performing *in vivo* studies as a comparison with *in vitro* assays.

II. L5 Binding

Analysis of the 5S RNA mutants tested showed that all were capable of binding L5 when injected into oocytes. These data are compatible with the *in vitro* binding data for these mutants presented in Appendix I, where the majority of mutants bound to L5 with near wild-type affinity (Q. You, W. Q. Zang, and P. J. Romaniuk, in prep.). Mutants most severely impaired for L5 binding *in vitro* were 27-32 and 45-54 (K_a 0.52 and 0.54 relative to oocyte-type) located in helix III. Both of these mutants were able to bind L5 *in vivo*. These results taken together suggest that the requirements of 5S RNA for L5 binding are less stringent than for TFIIIA binding. Similar findings were also reported by Guddat *et al.* (1990), where a series of mutant 5S RNAs containing internal deletions were expressed in oocytes. All of these deletion mutants were able to bind L5 to some degree, but none were able to bind TFIIIA (although this was analysed by immunoprecipitation).

Interestingly, the bulged cytidine at position 63 is not required for 5S RNA binding to L5 in *Xenopus*. In *E. coli*, where there are three 5S RNA-binding proteins, the equivalent residue at position 66 is highly important for L18 binding (Peattie *et al.*, 1981; Christiansen *et al.*, 1985). However, this bulged nucleotide was necessary for incorporation into the 60S ribosomal subunit in *Xenopus* (see below). Thus, although it has been proposed that the single eukaryotic 5S RNA-binding protein evolved from fusion of the genes encoding two or three of the prokaryotic 5S RNA-binding proteins (Nazar *et al.*, 1982), the results presented here suggest that the single eukaryotic 5S RNA-binding protein does not retain all of the properties of the three prokaryotic 5S RNA-binding proteins.

III. Assembly into 60S Ribosomal Subunits

Four of the mutant 5S RNAs tested were not precipitated with the anti-60S ribosomal subunit antiserum, indicating that these mutants were not assembled into stable 60S subunits. These results indicate that binding to L5 is not sufficient for ribosome assembly, since all of the mutants defective in ribosome assembly formed 5S RNPs *in vivo*. The mutants defective for ribosome incorporation identify four regions of the 5S RNA molecule that are essential for assembly into 60S ribosomal subunits, either by providing direct contacts for interaction with other ribosomal components, or by maintaining the necessary secondary or tertiary structure. It is of interest that each of these defective 5S RNAs contains changes or deletions of particular RNA structures which are known to be important in other RNA-protein interactions. Each of these is discussed below.

i. Mutant 10-13

Mutant 10-13, which contains nucleotide substitutions at positions 10, 11 and 13 in loop A was not incorporated into 60S ribosomal subunits. Subsequent studies using mutant 5S RNAs with individual mutations in loop A showed that nucleotide substitutions at positions 11 and 13 impair the molecule for ribosome assembly (L. A. Allison and P. J. Romaniuk, in prep.). As discussed above, loop A is thought

to perform a major role in the coaxial stacking of helices II and V (Christiansen *et al.*, 1987; Romaniuk, 1989; Baudin *et al.*, 1991). In the molecular model of Westhof *et al.* (1989), the colinearity of helices II and V is constrained by a triple base interaction between A13, G66 and U109. It therefore seems plausible that mutant 10-13 is defective for ribosome incorporation because of an altered conformation rather than due to a loss of sequence-specific contacts.

Base triple interactions have also been found to occur in other RNAs, where they are known to fix the relative orientations of helices, or to allow controlled relative motion of helices that are necessary for the biological function of the RNA (reviewed in Wyatt and Tinoco, 1993). Internal loops have also been shown to be important in protein binding to other RNAs. For example, *E. coli* ribosomal protein L1 recognises an internal loop in 23S rRNA and its own mRNA (reviewed in Draper, 1989), and ribosomal protein S8 also binds a helix-loop-helix motif present in 16S rRNA and its own mRNA (reviewed in Draper, 1989; Wu *et al.*, 1994).

ii. Mutants $\Delta 49,50$ and $\Delta 63$

The secondary structure of 5S RNA from all species contains nucleotides bulged out of helical regions; in *Xenopus* these are at positions 49, 50, 63 and 83. Deletion of the bulged adenosines at positions 49 and 50, or of the cytidine at 63 resulted in RNA molecules defective for ribosome assembly. Deletion of the bulged adenosine at position 83 had no effect on ribosome incorporation, indicating not all bulged nucleotides are necessary for integration.

The bulged nucleotide corresponding to C63 also appears to be important for ribosome biogenesis or function in yeast: Strains expressing mutant 5S RNAs in which this nucleotide was deleted were not viable (Van Ryk *et al.*, 1992). As discussed above, the bulged nucleotide in *E. coli* corresponding to C63 is critical for binding ribosomal protein L18 (Peattie *et al.*, 1981; Christiansen *et al.*, 1985). When this bulged nucleotide in *E. coli* was mutated from an adenosine to cytidine, the resulting RNA was still able to bind L18, and to be assembled into 50S subunits (Meier *et al.*, 1986). This result suggests that the actual identity of the bulged residue is not important, but that it confers a conformational structure that is recognised by L18 or other ribosomal proteins. This is supported by the observation that the

identity of this bulged nucleotide varies among the major phylogenetic divisions: adenosine in aerobic bacteria and yeast; cytidine in animals; and uridine in plants (Peattie *et al.*, 1981). Bulged nucleotides are thought to introduce axial kinking in RNA duplexes, and nuclear magnetic resonance spectroscopy has shown that single base bulges are usually stacked within the helix (Lilley, 1995), further suggesting conformational features are responsible for protein recognition.

Bulged nucleotides have been found to be part of the RNA structure recognised by a growing number of proteins. For example, the bacteriophage R17 coat protein represses translation by binding a hairpin loop structure containing a bulged adenosine in the R17 RNA (Romaniuk *et al.*, 1987b; Wu and Uhlenbeck, 1987). This bulged adenosine could also be substituted with guanosine without affecting coat protein binding, and it was suggested that the bulged residue was intercalated into the helix (Wu and Uhlenbeck, 1987). Other examples of bulged nucleotides having a role in protein interactions are found in the binding of Ro protein to hY RNA (Pruijn *et al.*, 1991) and in the iron-responsive-element binding protein interaction with its cognate RNA (Jaffrey *et al.*, 1993; Henderson *et al.*, 1994).

One of the most studied RNA-protein interactions is that between the human immunodeficiency virus type 1 (HIV-1) regulatory protein, Tat, and a sequence at the 5' terminus of all HIV transcripts, known as the *trans*-activation-responsive region (TAR). The binding site for Tat on TAR RNA is defined by a uracil-rich trinucleotide bulge located on a hairpin stem. Essential residues for Tat recognition are one uracil within the bulge and two base pairs immediately above the bulge (Weeks and Crothers, 1991; reviewed in Gait and Karn, 1993). The HIV type 2 Tat protein (Tat-2) also recognises stem-loop structures in the HIV-2 TAR RNA. In this case, two dinucleotide bulges are responsible for protein recognition (Rhim and Rice, 1994).

The size of the HIV-1 TAR RNA bulge was shown to be important for recognition by Tat. Mutants with bulges of two or three residues bound with similar affinity, whereas bulges of one or four nucleotides bound with lower affinity (Weeks and Crothers, 1991). These workers proposed a model in which the presence of bulges of two or three uracil residues distorts the conformation of the sugar-phosphate backbone sufficiently to widen the major groove to allow interactive

access of amino acid side chains. This hypothesis is supported by studies with diethyl pyrocarbonate (DEPC) and $\text{Rh}(\text{phen})_2(\text{phi})^{3+}$, which can be used to assess the accessibility of regions of RNA to proteins. Results showed that positions abutting bulges of two or three nucleotides were readily accessible, whereas RNA with no bulges or one nucleotide bulges showed little or no reactivity to these probes (Weeks and Crothers, 1993; Neenhold and Rana, 1995).

It is thus possible that the adenosine dinucleotide bulge at positions 49 and 50 of 5S RNA allows binding of ribosomal proteins in helix III by widening the major groove, analogous to Tat binding TAR RNA. Support for this hypothesis comes from experiments in which 5S RNA was probed with $\text{Rh}(\text{phen})_2(\text{phi})^{3+}$, which promotes strand cleavage at accessible sites in the major groove. Sites of cleavage included helix III, opposite the dinucleotide bulge, but not at sites of single nucleotide bulges (Chow *et al.*, 1992). Deletion of the dinucleotide bulge would result in a perfect A-form RNA helix, with the major groove inaccessible for protein interactions, thereby preventing necessary associations for ribosomal subunit assembly.

iii. Mutant 96-101

The structure of the loop E region of 5S RNA has been the subject of much investigation, with several conformational models put forward. Although differing in details, all of the models propose the existence of non-canonical base pairs, of the A·A, U·U and A·G types (Andersen *et al.*, 1984a; Romaniuk *et al.*, 1988; Westhof *et al.*, 1989; Wimberly *et al.*, 1993). The model proposed by Wimberly and colleagues is shown in Figure 3-1B. The unusual structure of the loop provides a number of easily recognisable potential protein binding sites: the bulged guanosine at position 75 in the major groove; the convoluted backbone of the 5' strand; and the narrow groove widths (Wimberly *et al.*, 1993). Similar RNA loop motifs have subsequently been identified in many RNAs, including 28S rRNA (sarcin/ricin loop; Szewczak *et al.*, 1993), 16S and 23S rRNAs, the central conserved region of viroid RNAs and also in the self cleaving RNA of the hairpin ribozyme (reviewed in Wimberly, 1994). This motif has been hypothesised to play an important role in specifying the tertiary structure of RNAs or perhaps the quaternary structure of some intermolecular RNA interactions (Wimberly, 1994).

Mutant 96-101 was constructed to allow the formation of canonical Watson-Crick base pairs, extending helix IV through the loop E region to helix V (Romaniuk, 1989). As shown here, these changes resulted in a 5S RNA molecule defective for ribosome assembly. Mutant 73-76, which contains base substitutions on the 5' side of loop E which retain the single-stranded nature of the region, was competent for ribosome assembly. In addition, subsequent experiments using a mutation with base substitutions on the 3' side of loop E, which also retained the single stranded nature of the loop, showed that this mutant was competent for ribosome assembly (L. A. Allison and P. J. Romaniuk, in prep.). These results imply that the actual structure formed by the non-canonical base pairs, abolished in mutant 96-101, provide an important conformational feature for protein recognition, rather than specific sequence requirements.

The importance of non-canonical base pairing for protein recognition has also been demonstrated for the HIV regulatory protein Rev, which binds to the Rev-responsive element (RRE) found in certain viral mRNAs. An important structural feature of RRE RNA for Rev binding is a stem-bulge-stem structure, with G·G and G·A type non-canonical base pairs within the bulge (Bartel *et al.*, 1991). Analogous to the model for loop E in 5S RNA, the formation of these non-canonical base pairs predicts a single bulged uracil residue. Since an A·A pair was able to functionally replace the G·G pair, it was proposed that the non-canonical base pairs distort the sugar-phosphate backbone, allowing sufficient opening of the major groove for specific recognition (Bartel *et al.*, 1991; reviewed in Gait and Karn, 1993). This was also supported by molecular modelling studies (Le *et al.*, 1994).

iv. Functional implications

The results presented here do not address the ability of the 60S subunits containing the mutant 5S RNAs to function in protein synthesis. However, some correlations can be made with the results of other functional studies. Probing of 5S RNA within mouse 60S subunits and 80S ribosomes showed that regions of 5S RNA that were accessible were located in loops C and D, as well as the bulged nucleotide at position 83 (Holmberg *et al.*, 1992), suggesting that they do not interact directly with other ribosomal components. This correlates with the results presented

here, where mutations in these regions did not affect assembly into 60S subunits (although these results only show that the sequences of these loops are not essential: further studies by L. A. Allison and P. J. Romaniuk [in prep.] have shown that the single-stranded nature of loops B and C is essential for ribosome assembly). Loop C is also the region of 5S RNA which has been postulated to interact with tRNA (Erdmann, 1976), so would need to be exposed for this interaction. Probing of 60S subunits from rat with DEPC also identified an additional cleavage site at adenosine 11 (loop A; Lo and Nazar, 1982). Since this residue is contained within the mutation 10-13, which was defective in ribosome assembly, this provides further evidence that loop A is critical for maintenance of the tertiary structure of 5S RNA, rather than providing base-specific contacts for ribosome assembly.

These results also do not distinguish between the formation of 60S subunits and 80S ribosomes. Since the bulk of stored ribosomes in *Xenopus* oocytes are in the form of 80S ribosomes (Miller, 1974; Dixon and Ford, 1982b), binding to the 40S subunit may be critical for the stability of 60S subunits containing mutant 5S RNAs. It is of interest then, that mutations 10-13 and 96-101 both lie within regions of 5S RNA that have been shown to base pair with 18S rRNA *in vitro* (Sarge and Maxwell, 1991). It is possible that 5S RNA containing these mutations is assembled into 60S subunits, but that these subunits are not stable as they can not interact with 40S subunits.

The results presented in this chapter indicate that more than one region of the 5S RNA molecule is important for ribosome assembly, suggesting the RNA may interact with more than one other ribosomal component. The secondary/tertiary conformation appears to be more important than the nucleotide sequence, with structures such as bulges, loops and non-canonical base pairs providing opportunities for protein recognition.

CHAPTER 4

DISTRIBUTION OF 5S RNA WITHIN THE *XENOPUS* OOCYTE NUCLEUS

4.1 INTRODUCTION

To be assembled into ribosomes, stored 5S RNA must first be imported into the oocyte nucleus and targeted to the nucleoli, the sites of ribosome biogenesis. The mechanism by which 5S RNA is targeted to nucleoli and incorporated into assembling 60S subunits is largely unknown. In the preceeding chapter, the necessary sequences and conformational structures of 5S RNA for protein associations in the cytoplasm, and for assembly into ribosomal subunits were investigated. Four mutant 5S RNA molecules were found to be defective for ribosome incorporation. As discussed in Chapter 3, the defective ribosome incorporation observed for these mutants may have resulted from conformational changes within the 5S RNA structure, rendering it unable to perform interactions necessary for integration into the 60S subunit. Alternatively, the lack of ribosome incorporation may have resulted from an earlier step of the 5S RNA pathway, such as nuclear import or nucleolar targeting. All of the mutant 5S RNA molecules were previously shown to be capable of nuclear import (data presented in Appendix I; Allison *et al.*, 1993; L. A. Allison and P. J. Romaniuk, in prep.). To determine whether the deficiency in ribosome assembly was due to defective nucleolar targeting, and to gain insight into the

process by which 5S RNA is incorporated into the 60S ribosomal subunit, I have investigated the distribution of endogenous and exogenous wild-type and mutant 5S RNAs, as well as 5S RNA-protein associations within the nucleus of *Xenopus* oocytes.

In order to investigate the process by which 5S RNA is incorporated into 60S subunits, an understanding of the site and process of ribosome synthesis is first necessary. The following sections review the structure and function of the nucleolus, describe some common nucleolar components, and finally review how these components are targeted to the nucleolus.

I. Structure and Function of the Nucleolus

In eukaryotes, ribosome biogenesis takes place within a specialised organelle, the nucleolus. Mature ribosomes are synthesised via a series of ordered steps, the general outline of which is shown in Figure 4-1. The nucleolus is the most prominent feature of the interphase nucleus. Although not bound by a membrane, it is exceedingly well defined, and appears refractile when viewed with phase contrast microscopy. It consists of three main components when viewed at the electron microscopic level: the fibrillar centre, the dense fibrillar component and the granular component. The fibrillar centre, named for the presence of five nanometre fibrils, is usually spherical, and has a low electron density. It is surrounded by the dense fibrillar component, which is also made up of five to ten nanometre fibrils. These fibrils are densely packed, causing a higher electron opacity. The dense fibrillar component is in turn surrounded by the granular component, which consists of loosely packed granules approximately fifteen nanometres in diameter, which closely resemble cytoplasmic ribosomes (reviewed in Goessens, 1984; Hadjiolov, 1985; Wachtler and Stahl, 1993). The arrangement of these components within nucleoli is variable and dynamic, depending on the species, cell type and physiological state of the cell. Current models propose that the ultrastructural components of the nucleolus are not fixed, but are formed by the transcription and subsequent processing of rRNA molecules (Dundr *et al.*, 1995; Hozák, 1995; Shaw *et al.*, 1995).

In *Xenopus* oocytes the rRNA genes are selectively amplified, resulting in the formation of approximately 1500 nucleoli per nucleus (Brown and Dawid, 1968; Perkowska *et al.*, 1968). These extrachromosomal nucleoli consist of inner fibrillar cores, surrounded with a granular cortex, similar to those of somatic cell nucleoli (Thomas, 1972; Van Gansen and Schram, 1972). In many cells, including *Xenopus* oocytes, nucleoli have been located close to, or in contact with the nuclear membrane (Bourgeois and Hubert, 1988). This has been postulated to facilitate the nuclear export of mature ribosomal subunits from the nucleolus to the cytoplasm.

The function of each of the three main nucleolar components has been the subject of intensive research, and has been extensively reviewed (Hadjiolov, 1985; Scheer and Benevente, 1990; Warner, 1990; Fischer *et al.*, 1991a; Wachtler and Stahl, 1993; Risueño and Testillano, 1994; Hozák, 1995; Mélése and Xue, 1995). Early studies using short term labelling with tritiated uridine showed that the dense fibrillar component was labelled before the granular component, suggesting a precursor-product relationship between the two (Granboulan and Granboulan, 1965). The actual location of the rDNA and the site of rRNA transcription have been controversial for many years, with some workers advocating the fibrillar centres and others the dense fibrillar component. More recently, non-isotopic ultrastructural methods have revealed that rRNA transcription appears to occur mainly within the dense fibrillar component, or at the border between the dense fibrillar component and the fibrillar centres (Dundr and Raška, 1993; Schöfer *et al.*, 1993; Hozák *et al.*, 1994; Raška *et al.*, 1995).

Transcription of rDNA in eukaryotes yields a primary pre-rRNA transcript of 35-47S, depending on the species. The general arrangement of sequences within this transcript, from 5' to 3' are: 5' external transcribed spacer (5' ETS), 18S rRNA, internal transcribed spacer 1 (ITS1), 5.8S rRNA, internal transcribed spacer 2 (ITS2), 28S rRNA, 3' external transcribed spacer (3' ETS; reviewed in Eichler and Craig, 1994). Mature 18S, 5.8S and 28S rRNAs are produced by a series of endonucleolytic and exonucleolytic cleavages, which generally occur from the 5' to 3' end of the nascent transcript. The order and intermediates generated during processing varies among species, as well as with growth and development in the same species (reviewed in Eichler and Craig, 1994). For example, in *Xenopus* oocytes, two alternative rRNA processing pathways were shown to coexist in the same cell

(Savino and Gerbi, 1990). Detailed understanding of the mechanisms of the processing steps is currently limited. Proteins thought to be involved in processing include fibrillarin, NOP1, GAR-1, SSB1 and RNase MRP (reviewed in Eichler and Craig, 1994). In addition, a series of small nucleolar RNAs (snoRNAs) have been identified and proposed to function in rRNA processing (reviewed in Maxwell and Fournier, 1995). For example, in *Xenopus*, U3 snoRNA is necessary for cleavage of the 5' ETS segment (Mougey *et al.*, 1993), U3 and U8 both influence processing at the ITS1-5.8S boundary (Savino and Gerbi, 1990; Peculis and Steitz, 1993), and U8 is required for cuts on the 3' side of 5.8S rRNA and both ends of 28S rRNA (Peculis and Steitz, 1993). U22 (also called RNA Y) is necessary for 18S rRNA maturation (Tycowski *et al.*, 1994).

In addition to processing, pre-rRNAs are also modified, including base and ribose methylations and pseudouridylation. These modifications are non-randomly distributed within rRNA sequences, and are clustered at evolutionarily conserved, functionally important sites. Possible roles of these modifications include serving as recognition signals for processing sites, effectors of rRNA conformation, and binding sites for specific proteins that effect the assembly of ribosomal subunits (reviewed in Maden, 1990; Eichler and Craig, 1994).

The fourth ribosomal RNA, 5S RNA, is transcribed from genes that are not nucleolar-associated (reviewed in Hadjiolov, 1985). Thus, 5S RNA must first be targeted to the nucleolus for ribosome incorporation. In *Xenopus* oocytes, where 5S RNA is stored in the cytoplasm, it must first be imported into the nucleus. Similarly, the ribosomal proteins which are synthesised in the cytoplasm must also be imported into the nucleus and targeted to the nucleolus for ribosome assembly (Fig. 4-1).

The actual steps of rRNA processing and ribosomal subunit maturation that take place within each nucleolar component remain to be elucidated. As the rRNA is transcribed, it is immediately coated with ribosomal and non-ribosomal proteins (Kumar and Warner, 1972; Chooi and Leiby, 1981). Thus, when the primary pre-rRNA transcript is released from the DNA template it is already assembled in the form of a preribosomal particle. Two types of preribosomal particles were initially identified in HeLa cells: an 80S particle containing the 45S pre-rRNA, and a

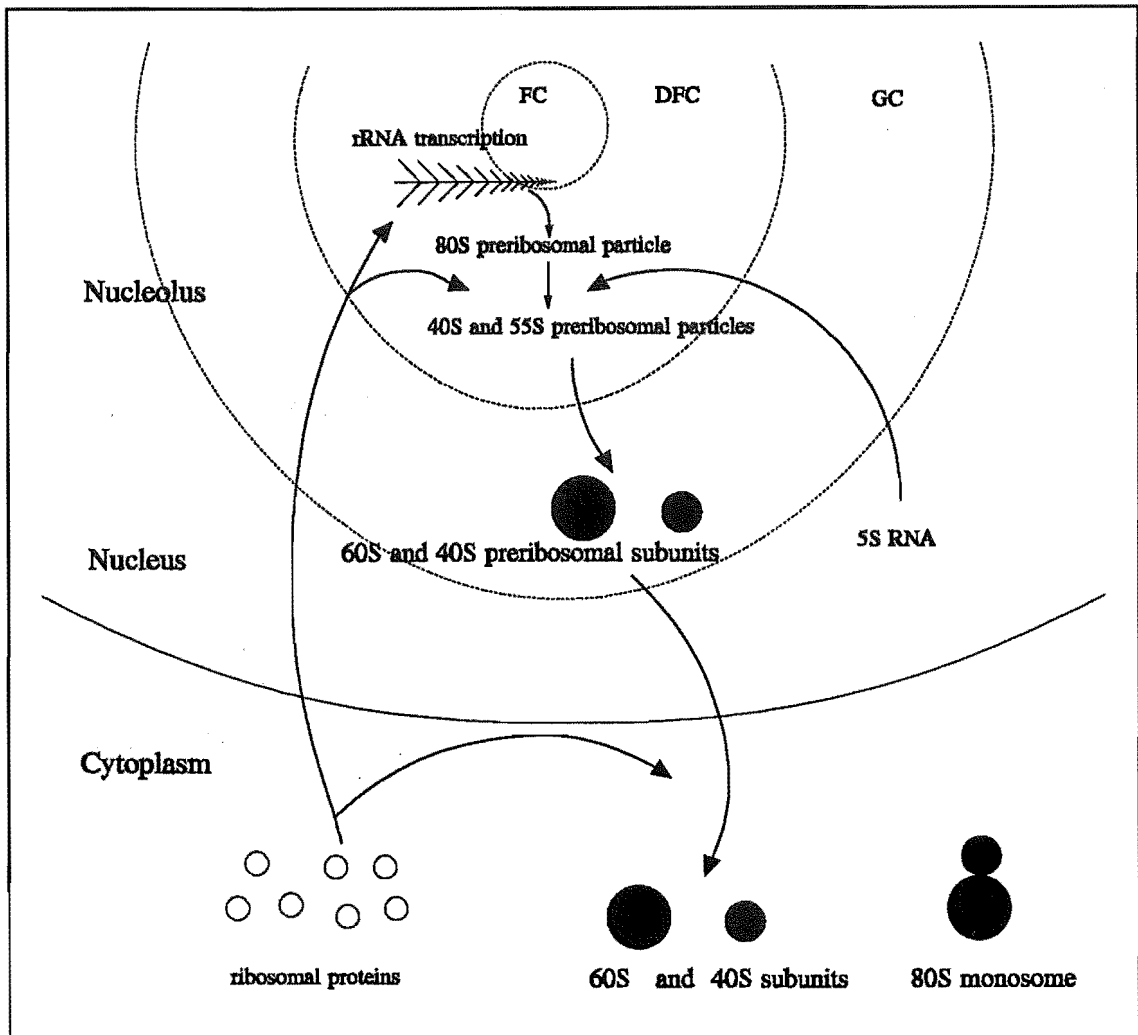


Figure 4-1 Ribosome biosynthesis in a typical eukaryotic cell. The diagram shows the various stages of ribosome synthesis and where each stage is thought to occur within the nucleolus. FC, fibrillar centre; DFC, dense fibrillar component; GC, granular component. rRNA transcription is shown to occur in the dense fibrillar component, at the border of the fibrillar centre. 5S RNA is incorporated in the dense fibrillar component, and is present in the 55S preribosomal particle. See text for references.

55S particle, containing 32S pre-rRNA, which is thought to be a precursor particle to the 60S ribosomal subunit (Warner and Soeiro, 1967). Similar complexes have since been identified in other species, including amphibian oocytes (Rogers, 1968; reviewed in Hadjiolov, 1985).

Recently, Shaw *et al.* (1995), using *in situ* hybridisation and parallel electron microscopy on pea root cells, revealed the presence of the external transcribed spacer in the dense fibrillar component only, whereas a probe to the entire 45S pre-rRNA showed a higher concentration in the surrounding granular component. Since excision

of the external transcribed spacer is one of the first pre-rRNA processing events, these results suggest that only the very early rRNA processing steps occur in the dense fibrillar component, with the remainder of maturation steps occurring in the granular component.

The finding that 5S RNA was present in 55S preribosomal particles indicated that 5S RNA is incorporated into ribosomes at an early stage of assembly (Knight and Darnell, 1967; Warner and Soeiro, 1967). This was further substantiated by recent ultrastructural mapping of 5S RNA, using *in situ* hybridisation, to both the granular component and the dense fibrillar component in HeLa cell nucleoli (Raška *et al.*, 1995).

II. Nucleolar Proteins

Since the dense fibrillar component is surrounded by the granular component, the site of 5S RNA integration into preribosomal subunits may not be readily accessible from the outside of the nucleolus. It could be proposed therefore that 5S RNA interacts with other nucleolar components which act as receptors, and provide a pathway to the site of integration into preribosomal particles. Many non-ribosomal, nucleolar proteins have been identified which are essential for ribosome biogenesis in eukaryotes, although their exact roles are unknown. None of these proteins have been shown to interact directly with 5S RNA, although some are associated with preribosomal particles containing 5S RNA (see below). Four nucleolar proteins which are known to have roles in ribosome biogenesis, and which may interact with 5S RNA/5S RNPs en route to the ribosome are described in detail below (summarised in Table 4-1), followed by a brief description of other proteins identified in the nucleolus to date.

Fibrillarin, named because of its location within fibrillar regions of the nucleolus, is a 34 kD protein found in all eukaryotes (Ochs *et al.*, 1985), including yeast, where it is called NOP1 (Schimmang *et al.*, 1989). Fibrillarin antibodies coprecipitate small nucleolar RNAs, including U3 (Lischwe *et al.*, 1985), U8 and U13 (Tyc and Steitz, 1989), as well as U14, U15, U16, U18, U20, U21, U22 and U24 (reviewed in Maxwell and Fournier, 1995). Mutations of *NOP1* in yeast

revealed roles in pre-rRNA processing, pre-rRNA methylation and ribosome assembly (Tollervey *et al.*, 1993).

B23 (also called nucleophosmin, numatrin, ribocharin, and NO38 in *Xenopus*) is a 38 kD phosphoprotein which has been localised to the dense fibrillar and granular components of the nucleolus in a variety of different cell types (Spector *et al.*, 1984; Hügler *et al.*, 1985; Schmidt-Zachmann *et al.*, 1987; Biggiogera *et al.*, 1989). B23 contains two domains rich in aspartic and glutamic acid, and the amino-terminal 124 amino acids show similarity to *Xenopus* nucleoplasmin. B23 binds to double and single stranded DNA and to rRNA via sequences at its carboxy-terminus (Wang *et al.*, 1994), exhibits RNA helix destabilising activity (Dumbar *et al.*, 1989), and has been shown to form stable oligomers *in vitro* and *in vivo* (Schmidt-Zachmann *et al.*, 1987; Chan and Chan, 1995). In further studies, B23 was shown to be associated with the induction of proliferation in B lymphocytes (Feuerstein and Mond, 1987), stimulated the activity of DNA polymerase- α (Takemura *et al.*, 1994), and also formed a complex with the transcription factor YY1, thus reversing transcriptional repression induced by YY1 (Inouye and Seto, 1994). B23, and an isoelectric variant initially termed ribocharin, have been found associated with 80S and 55S preribosomal components in HeLa cells and with 60S subunit precursor particles in *Xenopus laevis* oocytes, respectively (Hügler *et al.*, 1985; Yung *et al.*, 1985). Finally, B23 was recently shown to have ribonuclease activity, suggesting it may play a role in rRNA processing (Herrera *et al.*, 1995).

Another abundant nucleolar protein is nucleolin (also called C23; homologues in *Saccharomyces cerevisiae* and *Saccharomyces pombe* are called NSRI and GAR-2, respectively), which is present in all eukaryotes and has a molecular weight ranging from 92-105 kD (Hernandez-Verdun, 1991). Nucleolin has three distinct domains: an acidic amino-terminal region, four RNA-binding motifs and a glycine-rich carboxy-terminus (Lapeyre *et al.*, 1987; Caizergues-Ferrer *et al.*, 1989). Nucleolin is localised to the dense fibrillar and granular components of nucleoli (Biggiogera *et al.*, 1989), and binds to DNA (Olson *et al.*, 1983), including matrix-associated regions (Dickinson and Kohwi-Shigematsu, 1995), and to the external transcribed spacer of pre-rRNA via interactions with its RNA recognition motifs (Ghisolfi *et al.*, 1990; 1992; Gamberi *et al.*, 1994). In addition, other described activities of nucleolin include chromatin condensation, feedback regulation coupling rDNA transcription

Table 4-1 Nucleolar proteins involved in ribosome assembly

Protein	Nucleolar Component ^a	Activities/ Functions	Reference
Fibrillarin	FC and DFC	Association with snoRNAs; rRNA processing and methylation	Lischwe <i>et al.</i> , 1985 Tollervey <i>et al.</i> , 1993
B23/nucleophosmin/ NO38	DFC and GC	Associated with preribosomal subunits; Binds DNA and rRNA; Destabilisation of RNA helices; Induction of lymphocyte proliferation; Stimulation of DNA polymerase- α ; Ribonuclease activity; Shuttles between cytoplasm and nucleolus; Binds NLSs	Hügler <i>et al.</i> , 1985 Wang <i>et al.</i> , 1994 Dumbar <i>et al.</i> , 1989 Feuerstein and Mond, 1987 Takemura <i>et al.</i> , 1994 Herrera <i>et al.</i> , 1995 Borer <i>et al.</i> , 1989 Goldfarb, 1988
Nucleolin/C23	DFC and GC	Binds DNA; Binds pre-rRNA; Associated with matrix-associated regions; Shuttles between cytoplasm and nucleolus; Binds NLSs	Olson <i>et al.</i> , 1983 Ghisolfi <i>et al.</i> , 1992 Dickinson and Kohwi- Shigematsu, 1995 Borer <i>et al.</i> , 1989 Xue <i>et al.</i> , 1993
Nopp140/xNopp180	DFC	Shuttles between cytoplasm and nucleolus; Binds NLSs	Meier and Blobel, 1992 Meier and Blobel, 1990

^a FC, fibrillar centre; DFC, dense fibrillar component; GC, granular component. See text for references.

with rRNA processing, and binding to preribosomes containing 45S pre-rRNA (reviewed in Gerbi *et al.*, 1990). Also, the yeast homologues, NSR1 and GAR-2, are necessary for formation of 18S rRNA and 40S subunit assembly (Lee *et al.*, 1992; Gulli *et al.*, 1995).

Another nucleolar protein which was identified in rat is Nopp140 (nucleolar phosphoprotein of 140 kD; Meier and Blobel, 1990; 1992), a homologue of which has recently been cloned in *Xenopus*, called xNopp180 (Cairns and McStay, 1995).

Nopp140 contains a ten-fold repeated motif of highly conserved acidic serine clusters that contain an abundance of phosphorylation consensus sites for casein kinase II, and is localised to the dense fibrillar component (Schmidt-Zachmann *et al.*, 1984; Meier and Blobel, 1992).

In addition to being non-ribosomal, nucleolar proteins, the above three phosphoproteins, B23, nucleolin and Nopp140 share two further characteristics: each has been shown to shuttle between the nucleolus and cytoplasm (Borer *et al.*, 1989; Meier and Blobel, 1992), and each has been shown to specifically bind to the nuclear localisation sequences from either the SV-40 large T antigen or histone H2B (Goldfarb, 1988; Meier and Blobel, 1990; Lee *et al.*, 1991; Xue *et al.*, 1993; Szebeni *et al.*, 1995). It has therefore been suggested that these proteins act as chaperones, either targeting ribosomal proteins to the nucleolus, mediating ribosomal protein interaction with rRNA, or facilitating the export of ribosomal subunits to the cytoplasm. However, there is no direct evidence for any of these postulated functions.

Other proteins found in the nucleolus include those involved in rRNA transcription, such as RNA polymerase I and the transcriptional activator, UBF (upstream binding factor); proteins and RNPs necessary for rRNA processing, such as the product of the *Saccharomyces cerevisiae* *NOP4/NOP77* gene (Bergès *et al.*, 1994; Sun and Woolford, 1994) and 7-2 RNP (Reimer *et al.*, 1988); and a prolyl *cis-trans* isomerase (NP146), proposed to be involved in the assembly or folding of ribosomal proteins (Shan *et al.*, 1994). In addition, many proteins whose functions in this organelle are as yet unknown have been localised to the nucleolus. These include topoisomerase II (Fischer *et al.*, 1993); three Hox homeoproteins (Corsetti *et al.*, 1995); fibroblast growth factor 3 (Kiefer and Dickson, 1995); the interferon-inducible autoantigen, IFI 16 (Dawson and Trapani, 1995); protein p120, which plays a role in proliferation-associated nucleolar activity (Valdez *et al.*, 1994); and angiogenin, a potent inducer of angiogenesis (Moroianu and Riordan, 1994). In addition, many viral proteins have been found to be localised to the nucleolus of infected cells. The human T-cell leukaemia virus type I (HTLV-I) regulatory protein Rex (also called p27^{X-III}), the HIV Rev and Tat proteins, Semliki Forest virus capsid protein, and the herpes simplex virus regulatory protein, ICP27 all localise to nucleoli (Hauber *et al.*, 1987; Siomi *et al.*, 1988; Felber *et al.*, 1989; Jakob, 1993; 1994; Mears *et al.*, 1995). Two of these proteins, Rev and Rex, have been shown to specifically associate with

protein B23 *in vitro* (Fankhauser *et al.*, 1991; Adachi *et al.*, 1993; Szebeni *et al.*, 1995), and Rev has also been shown to shuttle between the nucleolus and cytoplasm (Meyer and Malim, 1994).

III. Nucleolar Targeting

The mechanism by which ribosomal proteins and 5S RNA are localised to nucleoli for ribosome assembly are currently unknown. Ribosomal protein L5 bound to 5S RNA is a precursor to ribosome assembly in a range of cell types (Steitz *et al.*, 1988; Allison *et al.*, 1991; Deshmukh *et al.*, 1993). In *Xenopus* oocytes, L5 is thought to bind 5S RNA in the cytoplasm and to mobilise it for nuclear import and ribosome assembly (Allison *et al.*, 1991). Targeting of 5S RNA to nucleoli could therefore be conferred by sequences or structures within the RNA or protein moieties of the RNP, or a combination of the two. The following sections review current knowledge on the process of targeting of proteins and RNAs to the nucleolus.

i. Nuclear import

The first requirement for the nucleolar targeting of molecules that are synthesised (for example, proteins) or stored (for example, 5S RNA) in the cytoplasm, is nuclear import. Nuclear import is an active process which occurs through the nuclear pore (reviewed in Stochaj and Silver, 1992; Agutter and Prochnow, 1994). Nuclear import of proteins is conferred by the presence of a nuclear localisation sequence (NLS) within the protein, of which there are two main types: short sequences of four to seven basic amino acids, as typified by the Simian virus 40 (SV-40) large T antigen NLS (Kalderon *et al.*, 1984a,b); and a bipartite NLS consisting of two sequences of basic amino acids separated by ten less conserved spacer amino acids, as typified by the nucleoplasmin NLS (Robbins *et al.*, 1991). By definition, NLSs are both necessary and sufficient for nuclear import: deletion of the NLS abolishes import of the resulting protein, and fusion of the NLS to a heterologous, cytoplasmic protein confers nuclear localisation on that protein.

The nuclear transport of most RNAs is thought to occur in association with proteins (for example, Mehlin *et al.*, 1992). Thus, nuclear import signals of RNA-protein complexes could reside either in the amino acid sequence of the protein, in the nucleotide sequence of the RNA, or in a combination of the two. As an example, the nuclear import of U1 small nuclear RNA (snRNA) requires the trimethylguanosine cap structure as well as the binding site for the common Sm proteins (Fischer and Lührmann, 1990; Hamm *et al.*, 1990). The nuclear import of U2 snRNA also requires the trimethylguanosine cap, but the import of U4 and U5 appears to have a less stringent requirement for this cap structure (Fischer *et al.*, 1991b).

Other factors involved in nuclear import include the nuclear pore targeting complex which consists of two subunits, importin- α and β (also called karyopherin α and β ; Radu *et al.*, 1995). Importin- α binds to NLSs, and together with importin- β , targets nuclear import substrates to the nuclear pore (reviewed in Simos and Hurt, 1995). Also involved in nuclear import is the GTP-binding protein Ran/TC4 (Ras-related nuclear protein), which forms a complex with a dimer of a protein called Ranip (Ran-interacting protein), and mediates the translocation of nuclear pore-docked karyophilic substrates into the nucleus (reviewed in Simos and Hurt, 1995).

In *Xenopus* oocytes, ribosomal protein L5 has been suggested to mobilise stored 5S RNA for import into the nucleus (Allison *et al.*, 1991). Preassembled 5S RNPs have been shown to be imported into the nucleus at a faster rate and to a higher extent than free 5S RNA, suggesting that binding to L5 is a prerequisite for nuclear import (K. J. Murdoch and L. A. Allison, submitted). Also, competition assays showed that nuclear import of 5S RNA was inhibited by the presence of excess P(lys)-BSA, a synthetic karyophilic protein which contains the SV-40 T antigen NLS, but not by excess U1 snRNA or U3 snoRNA (K. J. Murdoch and L. A. Allison, submitted). These results suggest that 5S RNA follows a similar pathway to many karyophilic proteins, rather than other RNA pathways for nuclear import.

ii. Nucleolar localisation

a. Proteins

Once inside the nucleus, at least two mechanisms can be envisioned whereby molecules are localised to the nucleolus: 1) nucleolar targeting occurs via a signal-dependent, possibly active mechanism, involving energy and nucleoplasmic receptors which function in transporting molecules to the nucleolus, or 2) since the nucleolus is not membrane bound, nucleolar localisation could occur by diffusion through the nucleoplasm and retention at nucleoli due to associations with other nucleolar components.

Initially, studies performed on the nucleolar targeting of viral proteins in mammalian cells supported the former mechanism. For example, a highly basic amino acid sequence at the amino-terminus of the Rex protein was shown to confer nucleolar localisation on *E. coli* β -galactosidase (Siomi *et al.*, 1988). Similar basic sequences were subsequently identified in the HIV proteins Rev and Tat, and in the herpes simplex virus protein ICP27, which could also direct nucleolar localisation when fused to cytoplasmic proteins (Kubota *et al.*, 1989; Cochrane *et al.*, 1990; Siomi *et al.*, 1990; Mears *et al.*, 1995).

However, subsequent targeting studies using cellular nucleolar proteins revealed that the requirements for nucleolar localisation were not so simple. For example, nucleolar localisation of nucleolin in both mammalian cells and *Xenopus* oocytes was found to be dependent not only on its NLS, but also on the RNA-binding domains and the glycine/arginine-rich domain (Créancier *et al.*, 1993; Heine *et al.*, 1993; Meßmer and Dreyer, 1993; Schmidt-Zachmann and Nigg, 1993). Since both the RNA-binding and the glycine/arginine-rich domains are necessary for specific and efficient binding of nucleolin to rRNA (Ghisolfi *et al.*, 1992), it was postulated that the nucleolar localisation of nucleolin was due to binding to rRNA. Similar results were also obtained for the yeast homologue of nucleolin, NSR1 (Yan and Mélése, 1993) and with B23/NO38 in *Xenopus* oocytes, where the carboxy-terminal region was found to be necessary, but not sufficient for nucleolar localisation (Peculis and Gall, 1992). This carboxy-terminal region was subsequently shown to be necessary for interaction with nucleic acids (Wang *et al.*, 1994). In

addition, the transcription factor UBF was found to require both the HMG-box 1 sequence, which binds to DNA, and the acidic tail region, which is speculated to interact with another transcription factor SP-1, for nucleolar localisation (Maeda *et al.*, 1992). Finally, although a specific domain of ribosomal protein S6 was recently shown to be essential for nucleolar localisation, this sequence was not sufficient to target the protein to the nucleolus (Schmidt *et al.*, 1995). It has therefore been suggested that there is no such thing as a nucleolar localisation sequence analogous to the NLS, but that nucleolar localisation is conferred by binding of functional domains to other nucleolar components.

Retention of molecules at the nucleolus due to the binding of functional domains implies the corollary that active functioning is necessary for localisation. This is supported by nucleolar localisation studies on UBF and B23. A reduction in the phosphorylation levels of the transcription factor UBF was shown to impair the ability of UBF to transactivate RNA polymerase I, and to also cause a redistribution of UBF between the nucleolus, nucleoplasm and cytoplasm in Chinese hamster ovary cells (O'Mahony *et al.*, 1992). Also, drugs which inhibited transcription or processing of rRNA caused translocation of B23 from the nucleolus to the nucleoplasm in HeLa cells. High doses of α -amanitin, inhibiting transcription of 5S RNA, also caused B23 translocation, however inhibition of protein synthesis had no effect (Yung *et al.*, 1985). These results suggest that ongoing rRNA transcription and processing, as well as the presence of 5S RNA, is required for nucleolar localisation of B23. Treatment with actinomycin D has also been shown to cause the redistribution of Rev from the nucleolus to the cytoplasm in mammalian cells, possibly due to the concomitant redistribution of B23 (Dundr *et al.*, 1995). Another requirement for nucleolar localisation of B23 appears to be GTP. HeLa cells in which cellular GTP pools were reduced by 70% with dehydrogenase inhibitors resulted in a shifting of B23 from the nucleolus to the nucleoplasm (Finch *et al.*, 1993). These data support the idea that localisation to nucleoli is via association of functional domains to other nucleolar components.

However, exceptions to the above findings were observed for some cellular proteins. Amino acids 31-35 from human angiogenin were sufficient to target non-nuclear proteins to the nucleolus in permeabilised endothelial cells (Moroianu and Riordan, 1994), and a seventeen amino acid sequence, plus the NLS, from protein

p120 was sufficient to target β -galactosidase to nucleoli (Valdez *et al.*, 1994). Closer inspection of the six proteins for which discrete nucleolar localisation sequences have been identified (Rex, Rev, Tat, ICP27, angiogenin and p120) reveals that three of these (Rex, Rev and p120) have been shown to specifically associate with the nucleolar protein B23 *in vitro* (Fankhauser *et al.*, 1991; Adachi *et al.*, 1993; Valdez *et al.*, 1994). In each case, the amino acids found to interact with B23 were within the sequence identified as the nucleolar localisation sequence.

It is thus tempting to speculate the following model for nucleolar localisation, whereby integral nucleolar proteins, such as B23, nucleolin and UBF, are anchored at the nucleolus due to the binding of functional domains to their respective substrates, for example, rDNA or rRNA. Other nucleolar proteins, such as angiogenin and p120, as well as viral proteins may then use the nucleolar localisation of the integral nucleolar proteins for their own localisation. This could be achieved by either of two mechanisms. In the first, these proteins are imported into the nucleus via their own NLS, and then subsequently localised to the nucleolus via interactions with proteins such as B23. Alternatively, since B23 has been shown to shuttle between the nucleolus and cytoplasm, it may have a more active role by binding these proteins in the cytoplasm, and directing them into the nucleus and to the nucleolus.

The utilisation of the "B23-directed" pathway of nucleolar localisation may provide more opportunities for the regulation of nucleolar localisation. For example, phosphorylation of B23 by casein kinase II was shown to enhance the binding of NLS peptides two-fold (Szebeni *et al.*, 1995). Since casein kinase II is present in the nucleolus, and its activity is enhanced in actively growing cells (Belenguer *et al.*, 1989), increased phosphorylation of B23 could result in increased localisation of protein p120 to nucleoli. Although B23 also binds to the SV-40 large T antigen NLS, it has a ten times higher affinity for Rev (Szebeni *et al.*, 1995). It seems possible then, that the HIV Rev protein has evolved to take advantage of a nucleolar localisation mechanism already occurring in host cells.

Since B23 is known to bind NLSs, its role could be extended to include the nucleolar targeting of other proteins, such as ribosomal proteins, including L5 and 5S RNPs. As both nucleolin and Nopp140 have also been shown to shuttle between the nucleolus and cytoplasm, and to bind NLSs (Borer *et al.*, 1989; Meier and Blobel, 1990; 1992; Xue *et al.*, 1993), these proteins may also perform a similar

function to that postulated for B23. Thus, a separate pathway may exist in cells for the nuclear import and subsequent nucleolar localisation of ribosomal/nucleolar proteins.

b. RNA

Very few studies have focussed on the mechanism of localisation of RNAs to the nucleolus. Nucleolar RNAs that are not transcribed on site include 5S RNA, the snoRNAs and 7-2 RNA (a component of RNase MRP). In addition, mRNAs encoding *c-myc*, *N-myc* and *myoD* were found to be localised to nucleoli in a number of diverse cell types (Bond and Wold, 1993). The functional significance of the localisation of these transcripts is not yet known.

Since RNAs are transcribed and processed in the nucleus (with the exception of 5S RNA, which is stored in the cytoplasm of amphibian oocytes), nucleolar localisation may occur by diffusion through the nucleoplasm and subsequent binding to other nucleolar components. Many snoRNAs have been found to contain remarkable sequence complementarities to rRNA (up to 21 nucleotides). This has led to the suggestion that these RNAs function in rRNA folding, maturation, or ribosomal RNP assembly via direct base pairing to rRNA (Bachellerie *et al.*, 1995). Furthermore, this sequence complementarity could also provide a mechanism for these molecules to be retained at the nucleolus. Alternatively, RNAs may bind either to proteins with which they are normally found associated; for example, U3 with fibrillarin or 5S RNA with L5, or with specialised receptor molecules in the nucleoplasm which target the RNAs to the nucleolus, perhaps using an active mechanism.

Experiments in cells depleted of the nuclear protein RCC1, which functions as a guanine nucleotide exchange factor for the GTPase, Ran/TC4, showed that newly synthesised U3 snoRNA is not localised to the nucleolus under these conditions (Cheng *et al.*, 1995). In addition, processing and maturation of pre-rRNA was impaired, suggesting necessary factors for these steps were also absent in these cells. These authors suggested the following model for intranuclear RNA transport. GTP-Ran/TC4, generated by RCC1, complexes with newly formed RNPs, promoting their diffusion through the nucleoplasm. Interactions with an RNP-specific GTPase-activating protein would then result in hydrolysis of GTP by Ran/TC4 and deposition

of the RNP. The location of the GTPase-activating protein would therefore determine the localisation of RNPs within the nucleoplasm. These findings also correlate with the above-mentioned results that the nucleolar localisation of B23 requires GTP (Finch *et al.*, 1993).

IV. The Present Investigation

To determine the mechanisms by which 5S RNA/5S RNPs are localised to nucleoli in *Xenopus* oocytes, the intranuclear distribution of endogenous 5S RNA was first established, before that of exogenously added 5S RNA. As described in the General Introduction, there are two types of 5S RNA synthesised in oocytes, oocyte-type and somatic-type, which differ by six nucleotides (Fig. 4-2). The predominant type of 5S RNA in oocytes is oocyte-type (Ford and Southern, 1973). Somatic-type 5S RNA has been shown to be imported into oocyte nuclei at a faster rate and to a greater extent than oocyte-type after injection into the cytoplasm, and more was incorporated into 60S ribosomal subunits (Allison *et al.*, 1995). It was therefore of interest to compare the abilities of these two molecules to be localised to nucleoli. To determine if the nucleolar localisation of ribosomal protein L5 paralleled that of 5S RNA, the distribution of radiolabelled, *in vitro* synthesised L5 within the oocyte nucleus was also analysed. To determine the requirements of 5S RNA for nucleolar localisation, the ability of a series of mutant 5S RNA molecules to be localised to nucleoli was tested. These results are compared with the results presented in Chapter 3 and with previous nuclear transport data. As a preliminary investigation to ascertain proteins with which 5S RNA is associated in the nucleus, immunoprecipitation assays were performed on nuclear and nucleoplasmic fractions of oocytes.

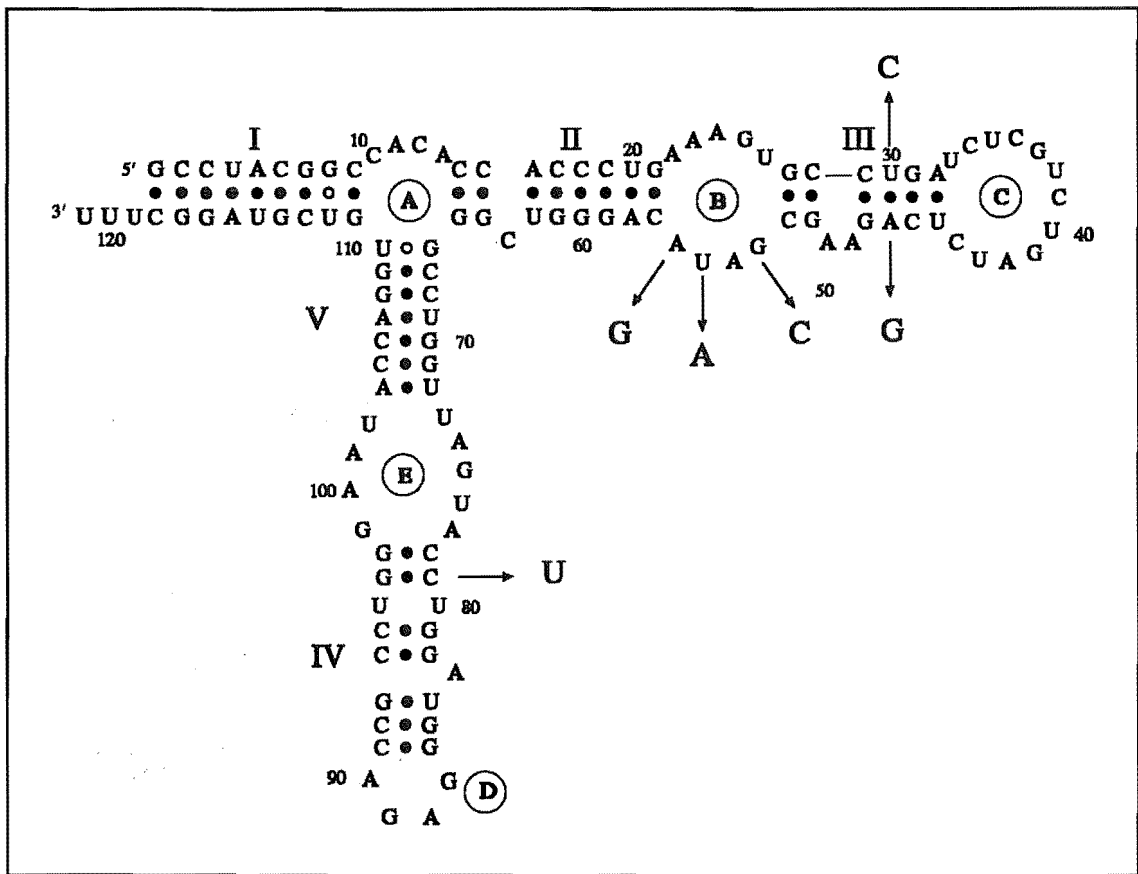


Figure 4-2 Secondary structure of *Xenopus laevis* 5S RNA showing somatic-specific substitutions. Nucleotides indicated by arrows, at positions 30, 47, 53, 55, 56 and 79 are those present in somatic-type 5S RNA.

4.2 RESULTS

I. Nucleolar Localisation of Endogenous 5S RNA

To investigate the nucleolar localisation of 5S RNA, a biochemical fractionation assay was utilised to isolate oocyte nucleoli, as described in Peculis and Gall (1992). Nuclei were manually dissected and broken open by sonication. The sonicated preparation was then centrifuged at 13 000 rpm to pellet the nucleoli. This procedure does not yield a pure preparation of nucleoli, since fragments of nuclear membrane, chromosomes and other insoluble aggregates are also pelleted under these conditions (Peculis and Gall, 1992).

The presence of intact nucleoli in the pellet fraction was confirmed by two means: detection of the nucleolar protein B23/NO38 and of 18S and 28S rRNA. Proteins were extracted from cytoplasmic, nucleolar and nucleoplasmic fractions from stage V oocytes and separated by SDS-PAGE. B23/NO38 was detected by immunoblotting with the anti-NO38 antibody, No-185 (Schmidt-Zachmann *et al.*, 1987). Results shown in Figure 4-3A reveal that all of the B23/NO38 was present in the nucleolar fraction (lane 2), consistent with previous localisation studies of this protein (Schmidt-Zachmann *et al.*, 1987; Peculis and Gall, 1992). In parallel experiments, total RNA was extracted from nucleolar and nucleoplasmic fractions, separated by agarose gel electrophoresis and analysed by northern blotting using a *Xenopus* rDNA probe. The results of this assay show that all of the rRNA was present in nucleolar fractions (Fig. 4-3B, lanes 1 and 3). These results indicate that all of the extrachromosomal nucleoli were pelleted by the centrifugation.

Since the genes encoding 5S RNA are not associated with nucleoli, it was possible that, unlike the other rRNAs, the distribution of 5S RNA in the nucleus would not be entirely nucleolar. Thus, I next determined the distribution of endogenous 5S RNA within the nucleus of oocytes. Total RNA was extracted from nucleolar and nucleoplasmic fractions, separated by agarose gel electrophoresis and analysed by northern blotting using an antisense 5S RNA probe, which binds specifically to 5S RNA (see Chapter 5). Analysis of stage V oocytes from three

different batches of oocytes revealed that on average, only 33% of endogenous, nuclear 5S RNA was associated with nucleoli (data not shown). Since the rate of ribosome assembly has begun to slow down by this stage of oogenesis, the distribution of 5S RNA within the nucleus was determined at various stages throughout oocyte development. Figure 4-4 shows the nucleolar localisation of endogenous 5S RNA from stage II to V oocytes. The large amount of 5S RNA present in stage II nuclei (lane 2) reflects the high rate of synthesis of 5S RNA in previtellogenic oocytes (Ford, 1971). Bands from this experiment were quantified by scanning densitometry, and the percentages of nucleolar localisation are presented in Table 4-2. Similar patterns of nucleolar localisation were observed in other experiments with different batches of oocytes (data not shown). The percentage of 5S RNA in the nucleus that is associated with nucleoli peaked at 40% in stage IV oocytes, coinciding with the peak of ribosome synthesis. These results indicate that throughout oogenesis there is a pool of 5S RNA within the nucleus that is not localised to the nucleoli.

II. Nucleolar Localisation of Exogenous 5S RNA

The large amount of 5S RNA not associated with nucleoli in the nucleus could represent newly synthesised 5S RNA which is en route to the nucleoli or cytoplasm. To test this, and to determine if exogenously added 5S RNA shows a similar nuclear distribution to endogenous 5S RNA, ^{32}P -labelled 5S RNA was microinjected into the cytoplasm of oocytes, thereby mimicking stored 5S RNA. The oocytes were incubated for 20 hours to allow nuclear import and nucleolar accumulation. The RNA was then extracted from cytoplasmic, nucleolar and nucleoplasmic fractions and analysed by PAGE and autoradiography. Figure 4-5 shows that oocyte-type 5S RNA is distributed similarly to endogenous 5S RNA within the nucleus. On average, 33% of the microinjected 5S RNA that had been imported into the nucleus was associated with nucleoli (lanes 1 and 2). Somatic-type 5S RNA, however, showed a different distribution pattern, with 70% of the RNA in the nucleus associated with nucleoli (lanes 3 and 4). This pattern was highly reproducible in many batches of oocytes, and did not appear to depend on the

Figure 4-3 Distribution of the nucleolar protein B23/NO38 and of rRNA within the *Xenopus* oocyte nucleus. (A) Distribution of B23/NO38. Nucleolar (No) and nucleoplasmic (Np) fractions were prepared from 50 isolated nuclei from stage V oocytes, by sonication and centrifugation to yield nucleolar pellets and supernatant (nucleoplasmic) fractions. Cytoplasmic fractions (Cy) were prepared from five enucleated oocytes only. Proteins were extracted from each fraction, separated by SDS-PAGE, and the subcellular distribution of B23/NO38 visualised by immunoblotting with an anti-NO38 antibody. (B) Distribution of rRNA. Nucleolar (No) and nucleoplasmic (Np) fractions from 40 oocytes were prepared as described in part (A). Total RNA was extracted from these fractions, separated by agarose gel electrophoresis and the distribution of rRNA determined by northern blotting using a labelled *Xenopus* rDNA probe (pXlr101A). The blot shows replicate samples of each fraction.

Figure 4-4 Nucleolar localisation of endogenous 5S RNA throughout oogenesis in *Xenopus* oocytes. Nucleolar (No) and nucleoplasmic (Np) fractions were prepared from 40 isolated nuclei by sonication and centrifugation. Total RNA was extracted and separated by agarose gel electrophoresis. The distribution of 5S RNA was determined by northern analysis using an antisense 5S RNA probe. Oocyte stages II to V (Dumont, 1972) are indicated.

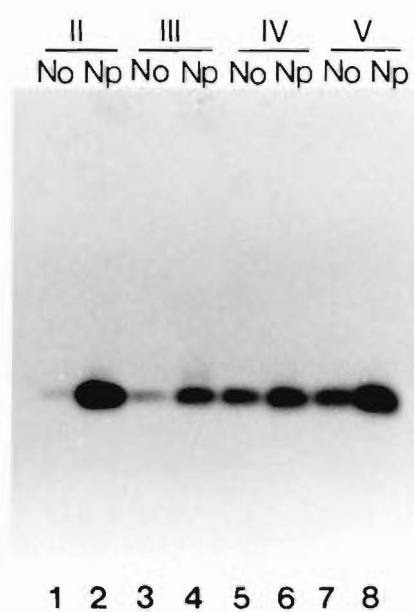
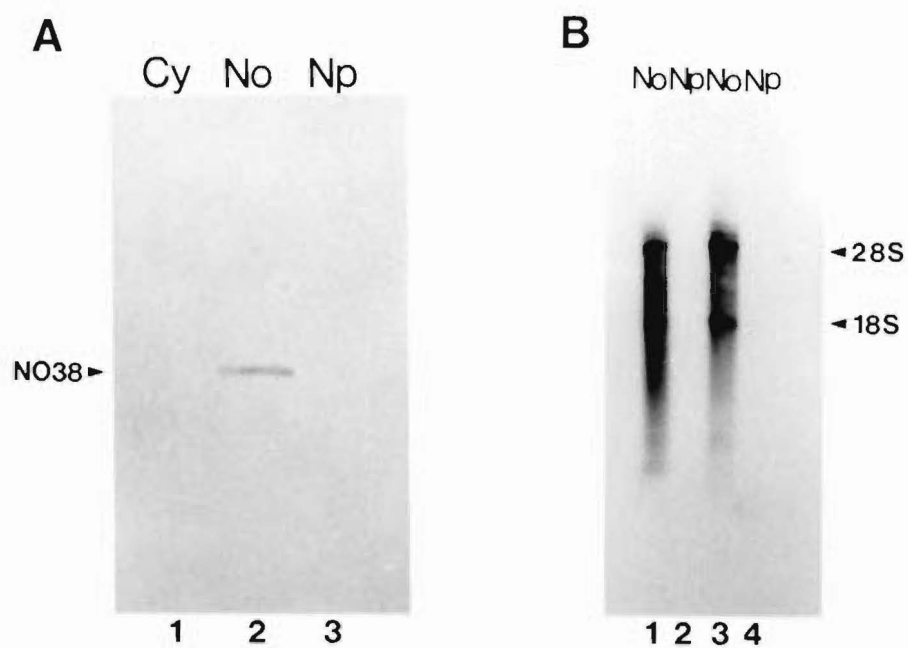


Table 4-2 Nucleolar localisation of 5S RNA throughout oogenesis

Stage of Oogenesis ^a	% Nucleolar Localisation ^b
II	6.3
III	21.6
IV	40.8
V	34.4

^a Oocyte stages according to Dumont (1972).

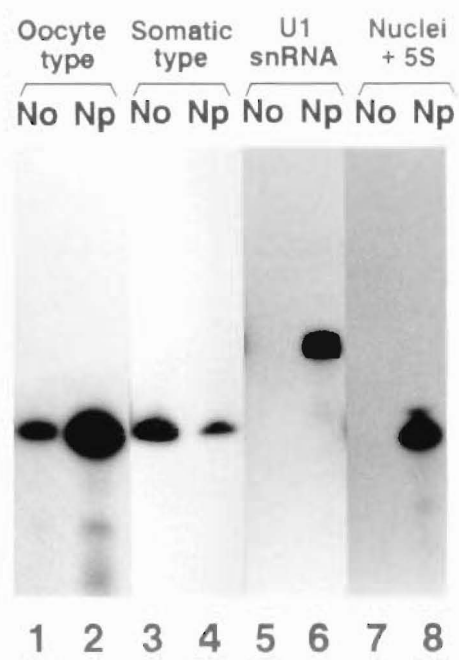
^b % Nucleolar localisation indicates the percentage of endogenous 5S RNA within the nucleus associated with nucleoli.

amount of RNA injected (data not shown). As a control, microinjected U1 snRNA was shown to be confined to nucleoplasmic fractions in most assays (lanes 5 and 6), although in a few experiments some U1 (approximately 10%) was detected in nucleolar pellets (data not shown). This was attributed to U1 being assembled into "B snurposomes", large granules present in amphibian oocyte nuclei that are thought to function in mRNA splicing (Wu *et al.*, 1991). In contrast to U1, U3 snoRNA showed a predominantly nucleolar distribution (data not shown).

To further confirm that the 5S RNA present in nucleolar fractions was not due to non-specific entrapment or sticking, ³²P-labelled 5S RNA was added to isolated nuclei after sonication treatment and the nucleoli pelleted by centrifugation. All of the added 5S RNA was found in the supernatant fraction (Fig. 4-5, lanes 7 and 8), indicating that the 5S RNA in nucleolar fractions is due to specific associations with nucleoli.

The results from these biochemical fractionation assays were confirmed using *in situ* localisation studies with semi-thin sections of oocytes. For these assays, ³³P-labelled RNAs were injected into the cytoplasm of oocytes, which were incubated

Figure 4-5 Nucleolar localisation of exogenous RNAs. ^{32}P -labelled oocyte-type 5S RNA, somatic-type 5S RNA or U1 snRNA were injected into the cytoplasm of stage V oocytes as indicated and incubated overnight. Nucleolar (No) and nucleoplasmic (Np) fractions were prepared from ten isolated nuclei by sonication and centrifugation. Total RNA was extracted from each fraction and analysed by 8 M urea/8% PAGE and autoradiography. Nuclei + 5S, nuclei were dissected from uninjected oocytes and ^{32}P -labelled 5S RNA was added, followed by preparation of nucleolar and nucleoplasmic fractions and analysis as described above.



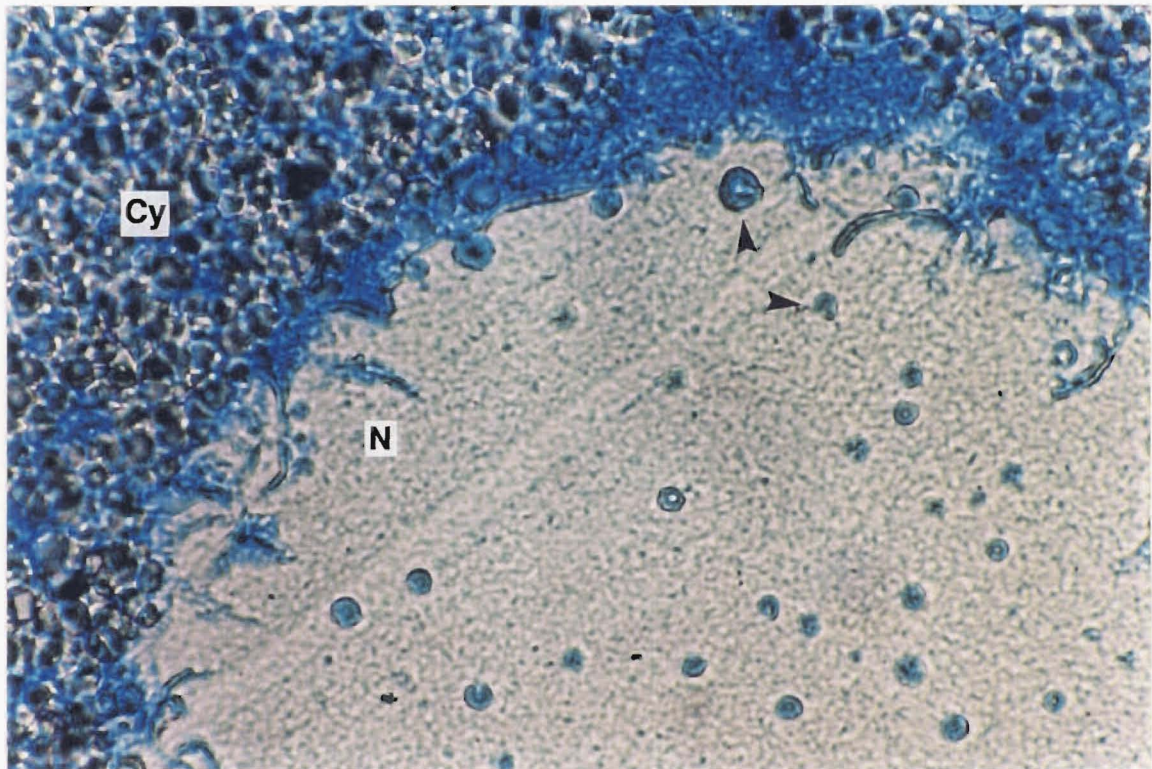
for 20 hours. The oocytes were then fixed, sectioned and subject to autoradiography. These results are shown in Figure 4-6. Panel (a) shows a non-injected oocyte nucleus, with very few silver grains, indicating low background levels. Oocyte-type 5S RNA showed a relatively homogeneous distribution within the nucleus, not dissimilar to that of U1 snRNA (compare panels b and c). Somatic-type 5S RNA, however, showed dramatic nucleolar localisation, consistent with the biochemical fractionation results above (panel d).

III. Nucleolar Localisation 5S RNA Mutants

To determine if specific sequences or secondary structures of the 5S RNA molecule are required for targeting to the nucleolus, the series of mutant 5S RNA molecules described in Chapter 3 were further assayed for their ability to be localised to nucleoli. Since the results described above indicated that the biochemical fractionation of nucleoli gave a more sensitive and quantitative assay of nucleolar localisation than analysis by semi-thin sections, this assay was used to assess the nucleolar localisation of the 5S RNA mutants. To avoid overloading the nucleus and saturation of putative nucleolar binding sites, ^{32}P -labelled mutant 5S RNAs were injected into the cytoplasm of stage V oocytes, rather than directly into the nucleus. The oocytes were incubated for 20 hours to allow nuclear import and nucleolar localisation, and the RNA extracted from nucleolar and nucleoplasmic fractions. The distribution of RNA within the nucleus was analysed directly by liquid scintillation counting, or by PAGE and autoradiography followed by quantification using scanning densitometry. Nucleolar localisation for each mutant was calculated as a percentage of the RNA in the nucleus. Therefore, the results do not reflect the differences in nuclear import which have previously been observed (Allison *et al.*, 1993), or possible differences in nuclear export, of the different mutants. Since there is slight variation between batches of oocytes, the nucleolar localisation of each mutant was compared with that of oocyte-type 5S RNA within the same batch of oocytes, and all mutants were tested in at least two different batches from different frogs.

The results from a selection of mutants are shown in Figure 4-7, and a summary of all the mutants tested, made relative to oocyte-type, is presented in

a



b

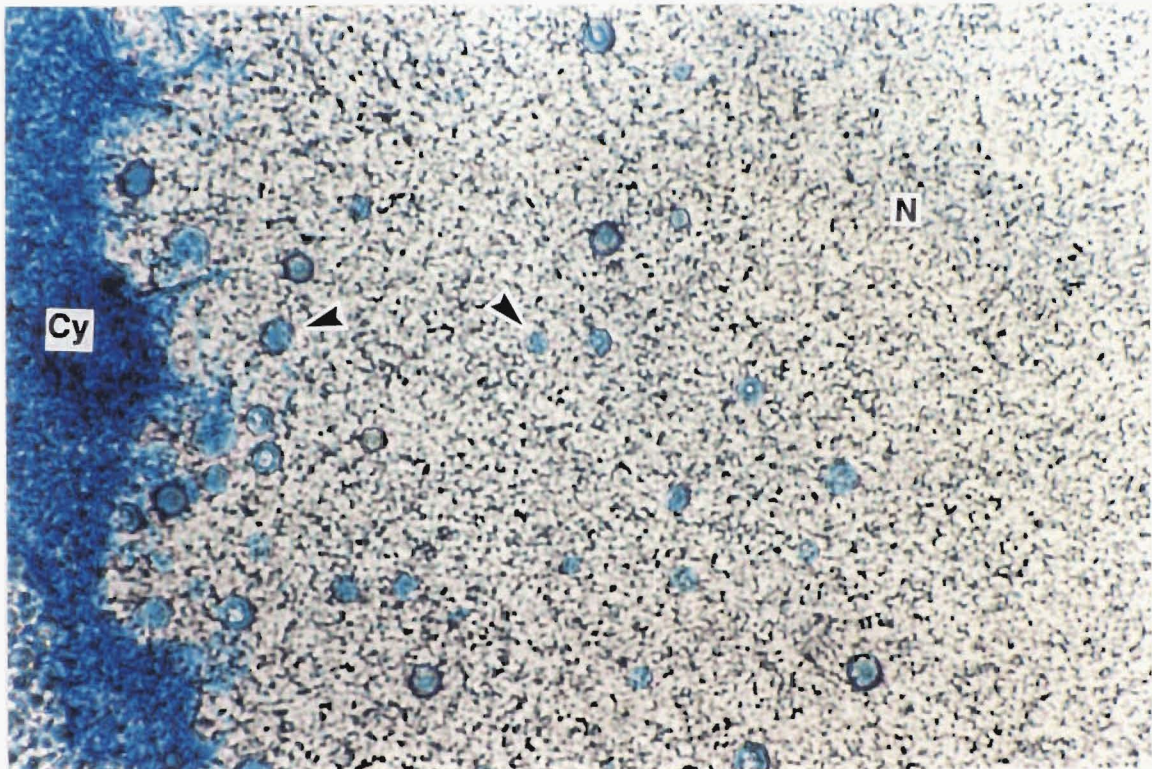
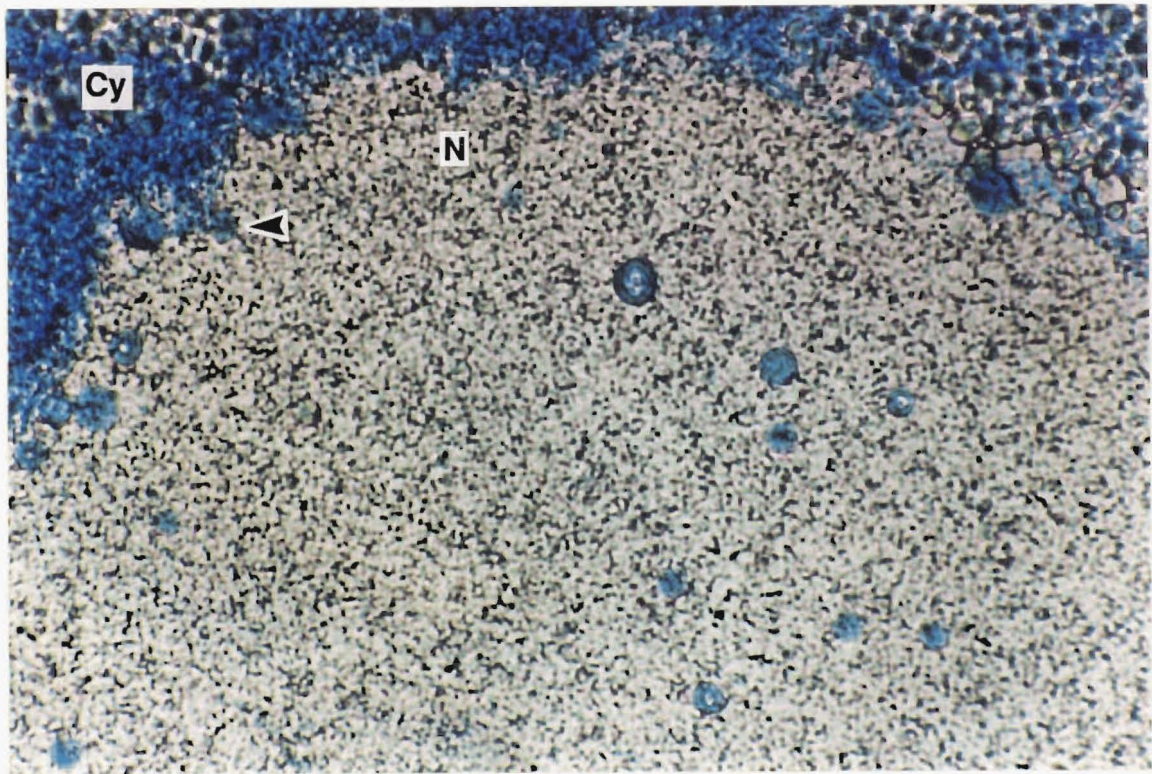
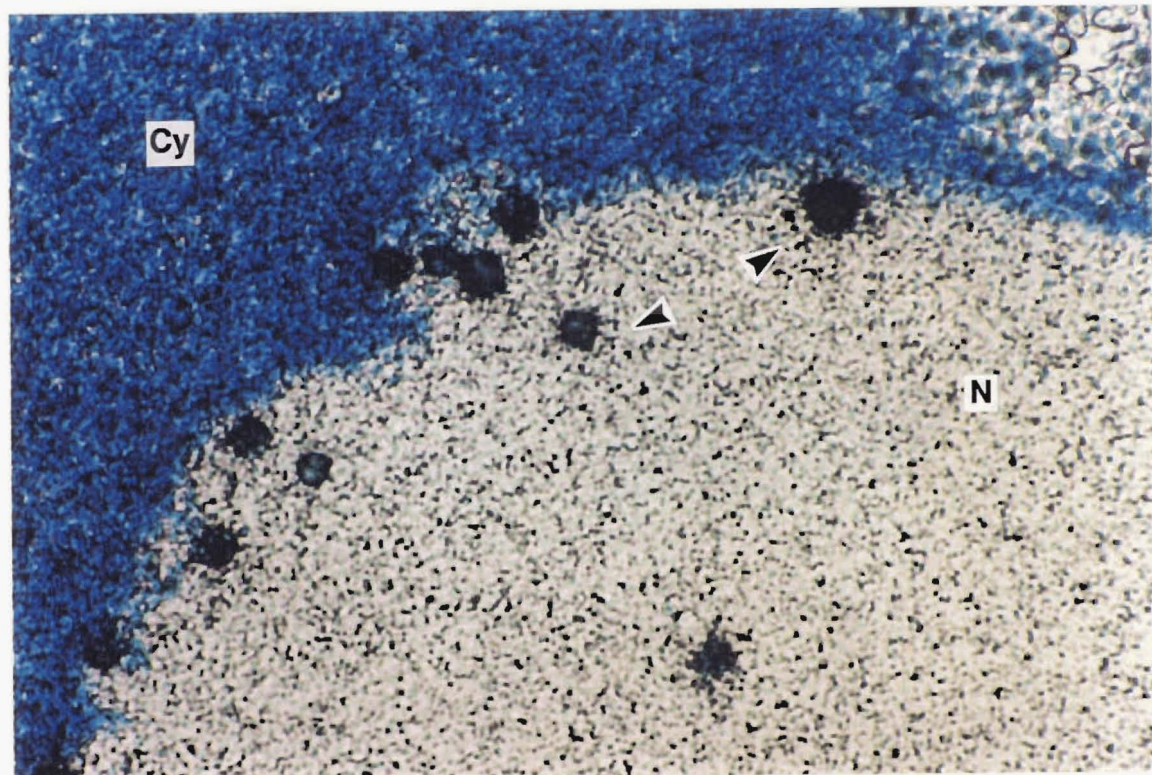


Figure 4-6 *In situ* nucleolar localisation of exogenous RNAs in *Xenopus* oocytes. ^{33}P -labelled RNAs were injected into the cytoplasm of stage V oocytes, which were incubated overnight. Oocytes were then fixed, sectioned at four to seven microns, subject to autoradiography and stained with Giemsa. (a) non-injected control oocyte; (b) oocyte injected with U1 snRNA;



d



(c) oocyte injected with oocyte-type 5S RNA; (d) oocyte injected with somatic-type 5S RNA. Cy, cytoplasm; N, nucleus; arrows indicate some of the extrachromosomal nucleoli. Bars 30 μ m.

Figure 4-8. The actual percentages of nucleolar localisation for each mutant are presented in Appendix II. None of the mutants tested were completely defective for nucleolar localisation using this assay, although there was variation in the amounts localised. The majority of 5S RNA mutants showed similar nucleolar localisation to oocyte-type 5S RNA, for example, mutant 27-32 (Fig. 4-7, lanes 3 and 4). However, a few mutants, for example, mutants with substitutions at positions 14-15 and 16-21 in helix II, showed lower levels of nucleolar accumulation (lanes 5, 6, 13 and 14); and other mutants, for example 57-62 and 95-98, showed higher amounts of nucleolar localisation than oocyte-type (Fig. 4-8).

Of particular interest were the four mutants which were shown to be defective for incorporation into the 60S ribosomal subunit. Mutant 10-13 showed a similar nucleolar localisation to oocyte-type (30.5%; Fig. 4-7, lanes 15 and 16), whereas mutant 96-101 showed slightly less localisation than oocyte-type (lanes 7 and 8). The two deletion mutants, $\Delta 49,50$ (lanes 11 and 12) and $\Delta 63$ (lanes 9 and 10), however, repeatedly showed much higher levels of nucleolar localisation (78.6% and 61.2%, respectively).

To confirm that these results did indeed represent nucleolar localisation, *in situ* localisation assays were also performed with a selection of the mutant 5S RNAs. Most of the mutants tested in this assay showed nucleolar localisation characteristics consistent with the biochemical fractionation results, for example, mutants 10-13 and 96-101 (Fig. 4-9, panels a and b). However, the two mutants, $\Delta 49,50$ and $\Delta 63$, which showed high nucleolar localisation according to the biochemical fractionation, did not show a corresponding nucleolar localisation in the *in situ* assays (Fig. 4-9, panels c and d). Rather, the distribution of these two mutants within the nucleus was very similar to oocyte-type 5S RNA. Silver grains appeared homogeneously distributed throughout the nucleus, including over nucleoli, and were not aggregated or associated with specific structures such as the nuclear envelope. These results imply that these two mutant RNAs are binding to other structures within the nucleus, resulting in their presence in nucleolar pellets. When ^{32}P -labelled $\Delta 49,50$ was added to isolated nuclei, followed by isolation of nucleoli and extraction of RNA, all of the added $\Delta 49,50$ was in the nucleoplasmic fraction (data not shown), similar to oocyte-type 5S RNA (Fig. 4-5, lanes 7 and 8). This shows that the

Figure 4-7 Nucleolar localisation of mutant 5S RNA molecules. ^{32}P -labelled oocyte-type (oocyte) or mutant 5S RNA molecules were injected into the cytoplasm of stage V oocytes, followed by an overnight incubation. Nucleolar (No) and nucleoplasmic (Np) fractions were prepared from ten isolated nuclei by sonication and centrifugation. Total RNA was extracted and analysed by 8 M urea/8% PAGE and autoradiography. Mutant designations refer to those areas of the molecule which were substituted or deleted (Fig. 3-3).

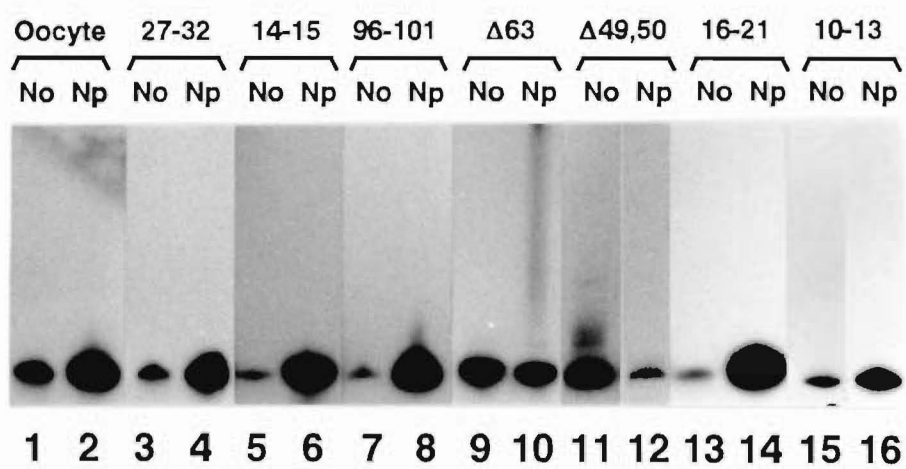
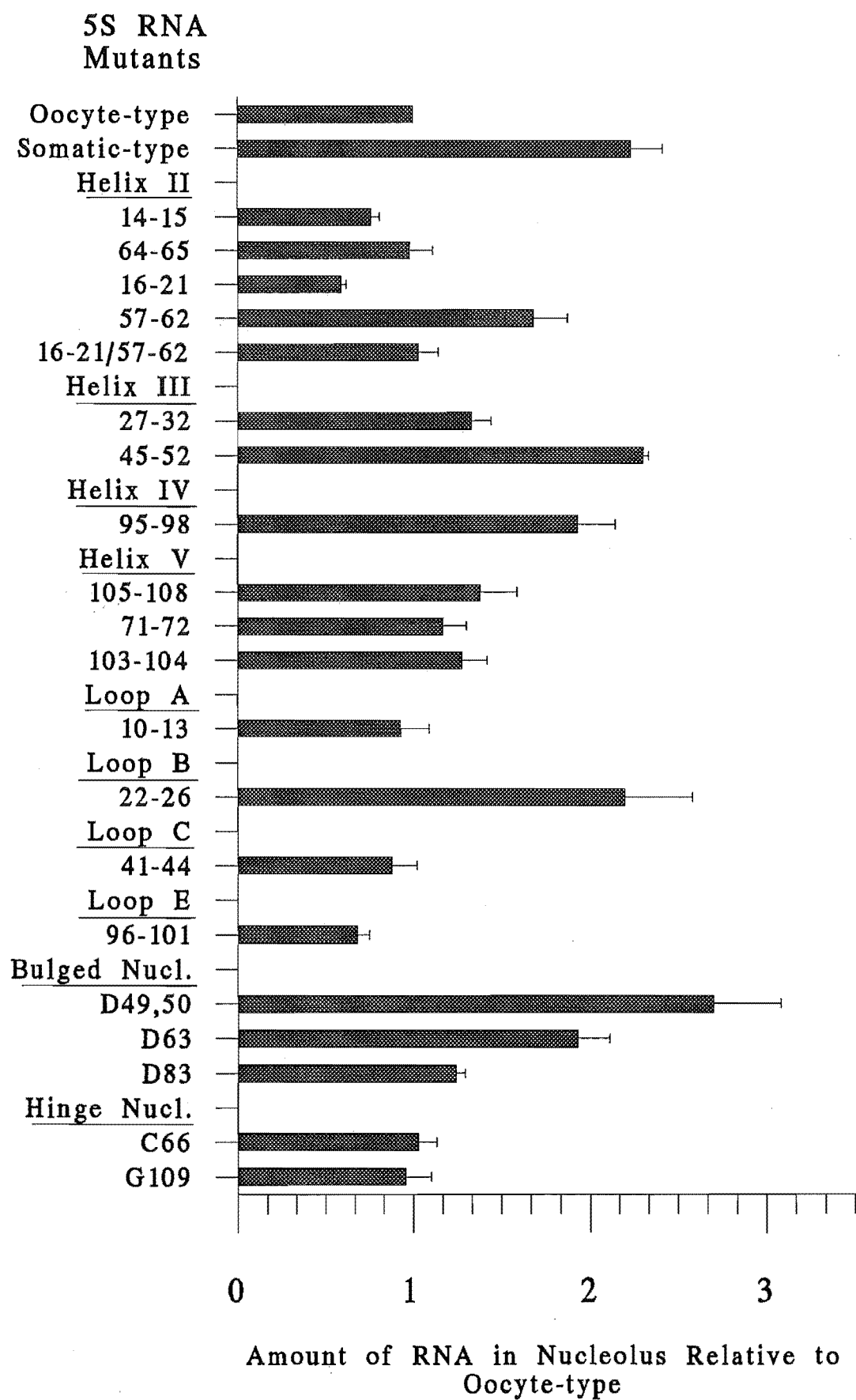


Figure 4-8

Figure 4-8 shows the results of the analysis of the data from the 1990s. The results show that the average number of children per woman is 2.1, which is slightly below the replacement level of 2.1. This suggests that the population is not growing, but is slowly declining.

Figure 4-8 Summary of nucleolar localisation of 5S RNA mutants. Nucleolar localisation was calculated as a percentage of the RNA in the nucleus (data presented in Appendix II), and expressed relative to the nucleolar localisation of oocyte-type 5S RNA within the same batch of oocytes. Mutant designations refer to those areas of the 5S RNA molecule which were substituted or deleted (Fig. 3-3).



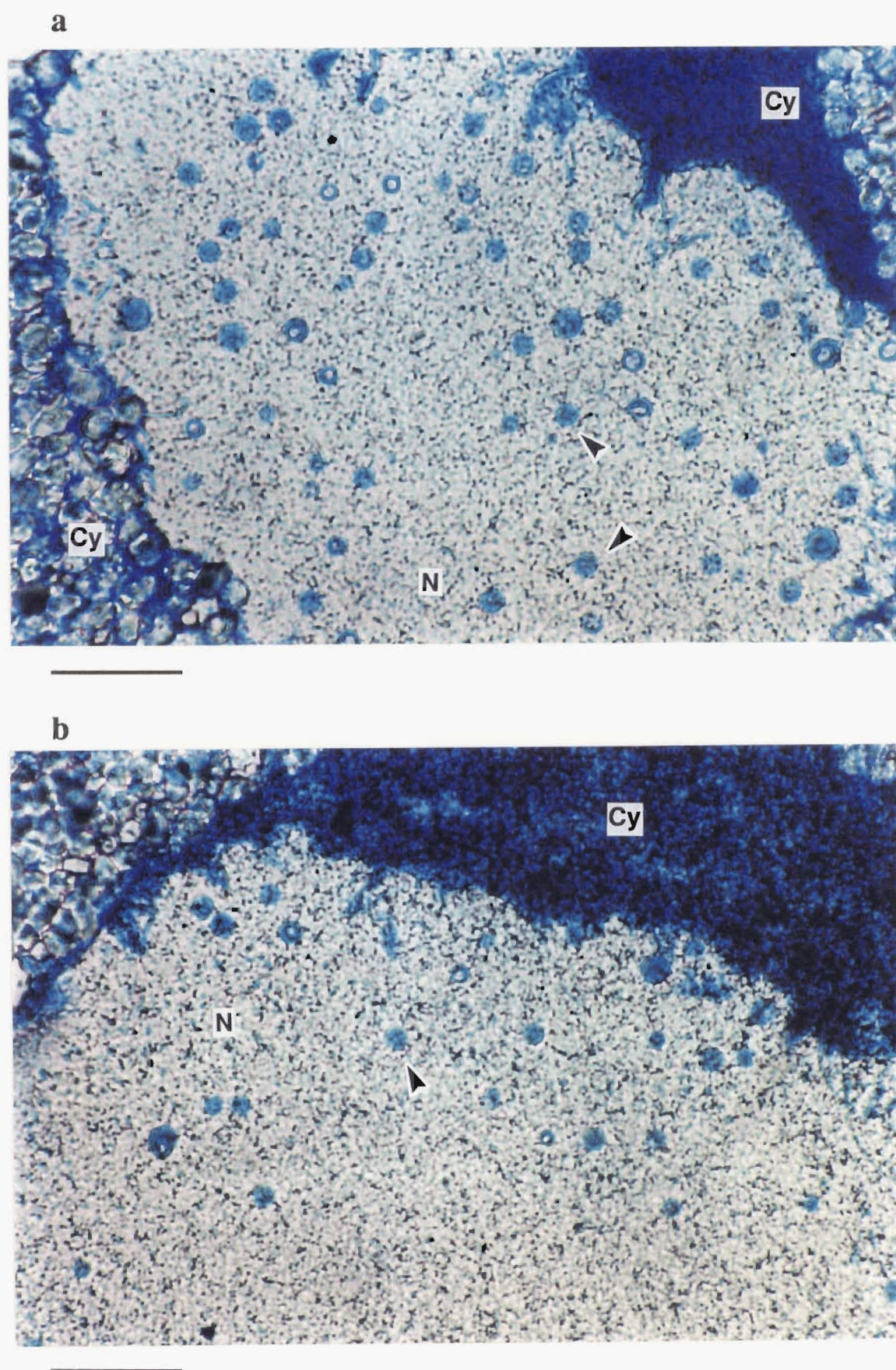
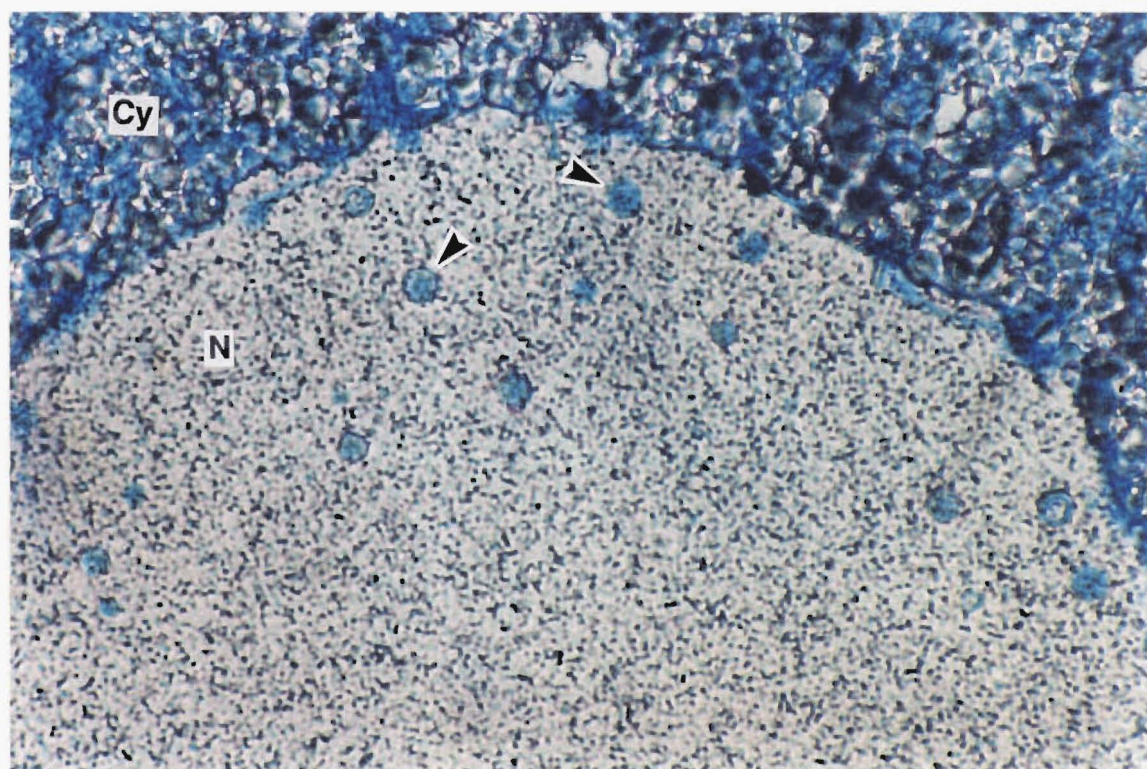
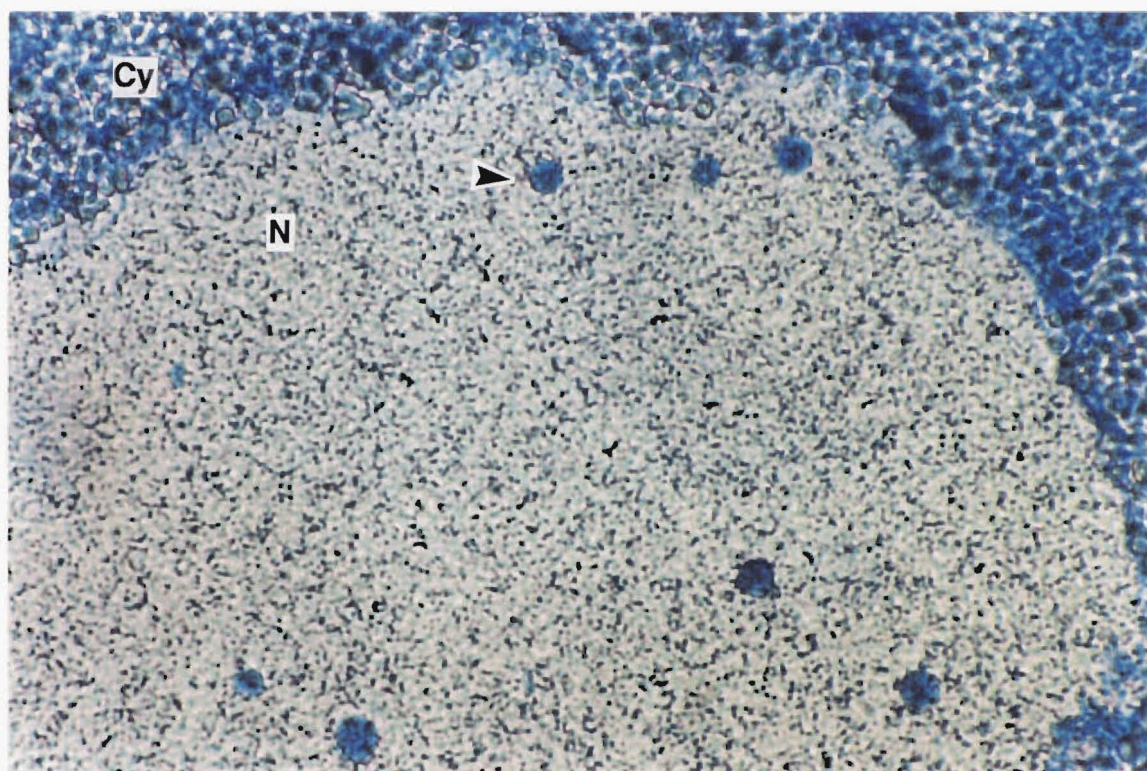


Figure 4-9 *In situ* nucleolar localisation of 5S RNA mutants. ^{33}P -labelled mutant 5S RNAs were injected into the cytoplasm of stage V oocytes, which were incubated overnight. Oocytes were fixed, sectioned at four to seven microns, subject to autoradiography and stained with Giemsa. (a) oocyte injected with mutant 10-13; (b) oocyte injected with mutant 96-101;



d



(c) oocyte injected with mutant $\Delta 49,50$; (d) oocyte injected with mutant $\Delta 63$.
Cy, cytoplasm; N, nucleus; arrows indicate some of the extrachromosomal nucleoli.
Bars 30 μm .

presence of $\Delta 49,50$ in nucleolar pellets was not due to some spontaneous interaction with nuclear components.

IV. Nucleolar Localisation of Ribosomal Protein L5

Since ribosomal protein L5 has been implicated in the transport of 5S RNA into the oocyte nucleus (Allison *et al.*, 1991; 1995; K. J. Murdoch and L. A. Allison, submitted) and has also been postulated to target 5S RNA to the nucleolus in HeLa cells (Steitz *et al.*, 1988), it was of interest to determine the nuclear distribution of this protein. ^{35}S -labelled L5 was synthesised using a rabbit reticulocyte lysate-coupled transcription-translation reaction, and this lysate mixture was injected directly into the cytoplasm of oocytes. After 20 hours incubation in the presence of cycloheximide to prevent incorporation of excess ^{35}S -methionine into oocyte proteins, nucleolar fractions were prepared as above, and the proteins extracted and analysed by SDS-PAGE and fluorography. Quantitation of bands indicated that 40% of L5 within the nucleus was nucleolar-associated (Fig. 4-10). As a control for the presence of cycloheximide, the distribution of 5S RNA was also assessed under the same conditions. The average oocyte-type 5S RNA nucleolar localisation in these assays was 23.5% (data not shown). This value is below the average nucleolar localisation observed for oocyte-type 5S RNA, but is not out of the range of values observed owing to oocyte variability (see Appendix II). Cycloheximide has also been shown not to affect the nucleolar localisation of B23 (Yung *et al.*, 1985).

The L5 localisation results were also confirmed using *in situ* localisation assays. Since the ^{35}S -labelled L5 was not purified from the unincorporated ^{35}S -methionine in the reaction mixture, controls were performed in which oocytes were injected with the product of a transcription-translation reaction which was primed with H_2O instead of template DNA. A typical section of such an oocyte is shown in Figure 4-11. Most of the ^{35}S -methionine was localised within the cytoplasm, with only a small number of silver grains in the nucleus, which appeared to be randomly distributed (panel a). Thus, the intranuclear distribution of injected ^{35}S -L5 (panel b) reflects mostly localisation of L5 protein and not background ^{35}S -methionine. These sections showed distinct accumulations of silver grains over

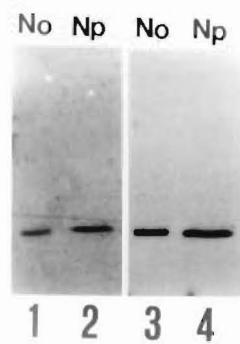


Figure 4-10 Nucleolar localisation of ribosomal protein L5. ^{35}S -labelled L5 was injected into the cytoplasm of stage V oocytes, which were then incubated overnight. Nucleolar (No) and nucleoplasmic (Np) fractions were prepared from 20 isolated nuclei by sonication and centrifugation. Proteins were extracted from each fraction and analysed by SDS-PAGE and fluorography. Lanes 1-2 and lanes 3-4 show replicates from experiments with different batches of oocytes.

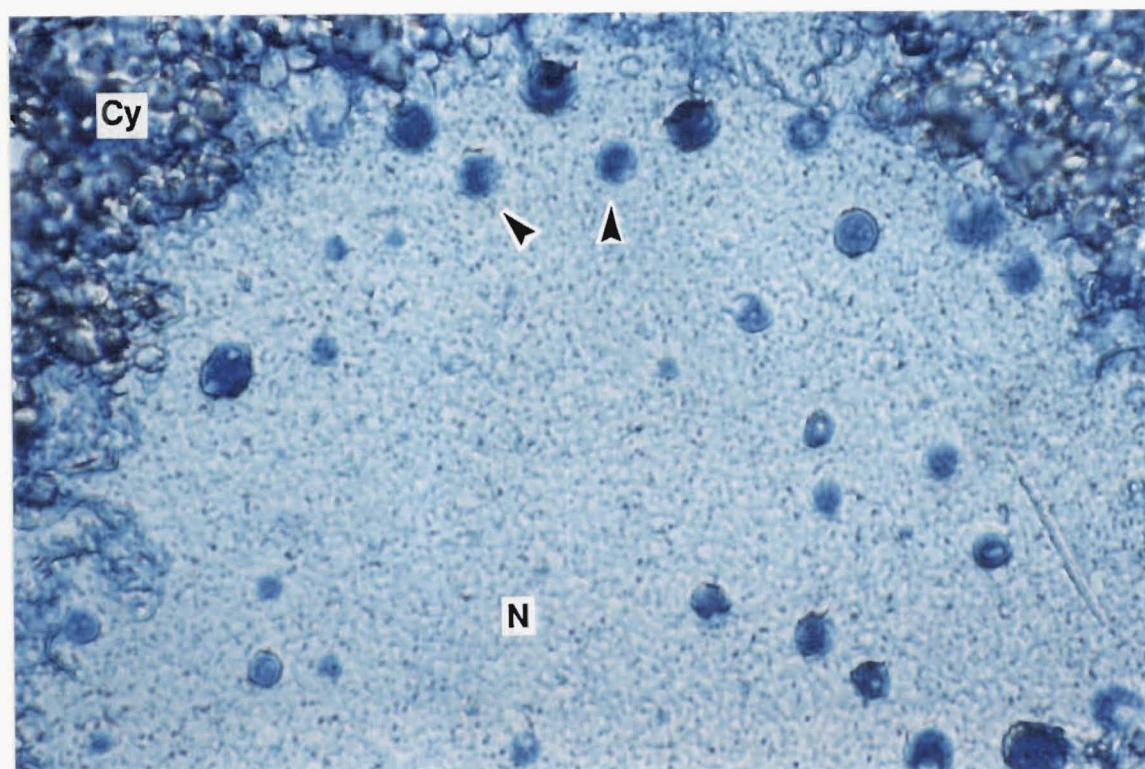
Figure 4-11 . *Endemic species of fish and amphibians*

13

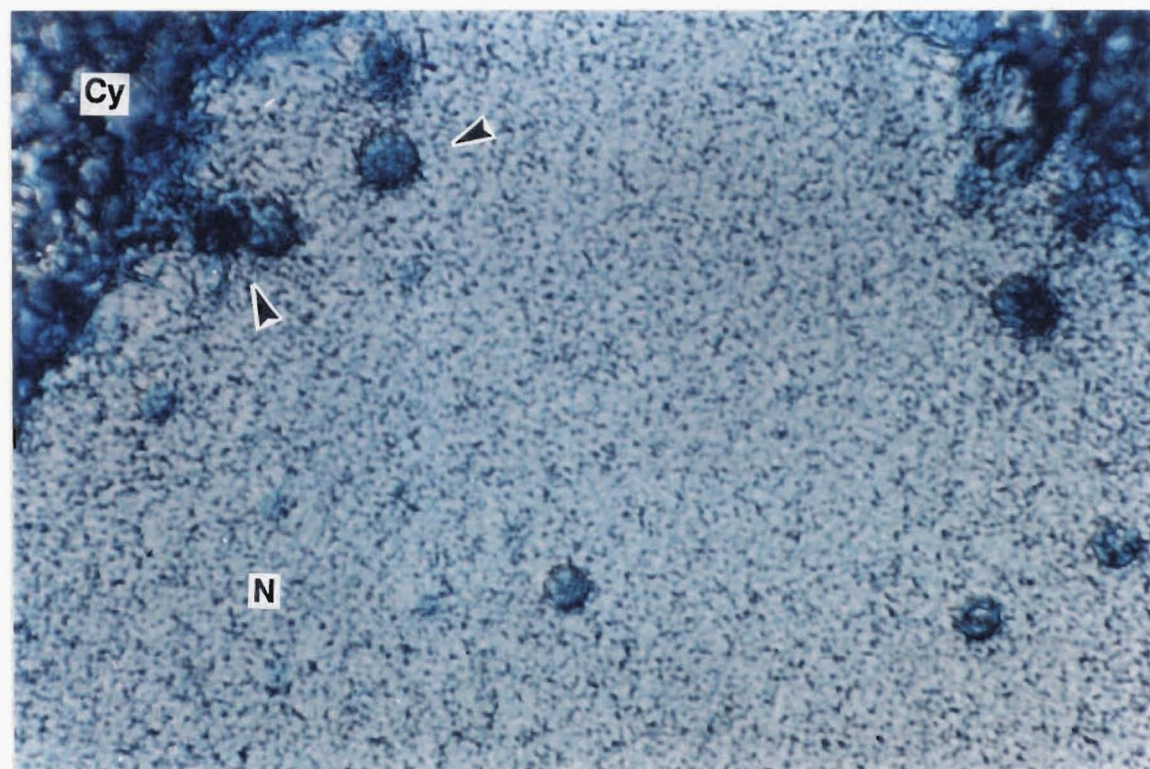
Figure 4-11 *In situ* nucleolar localisation of ribosomal protein L5. Stage V oocytes were cytoplasmically injected with the ^{35}S -labelled product of an *in vitro* transcription-translation reaction either primed with H_2O as a control (a) or with L5 template (b), and incubated overnight. Oocytes were then fixed and sectioned at five microns, subject to autoradiography and stained with Giemsa. Cy, cytoplasm; N, nucleus; arrows indicate some of the extrachromosomal nucleoli. Bars 30 μm .

a

91



b



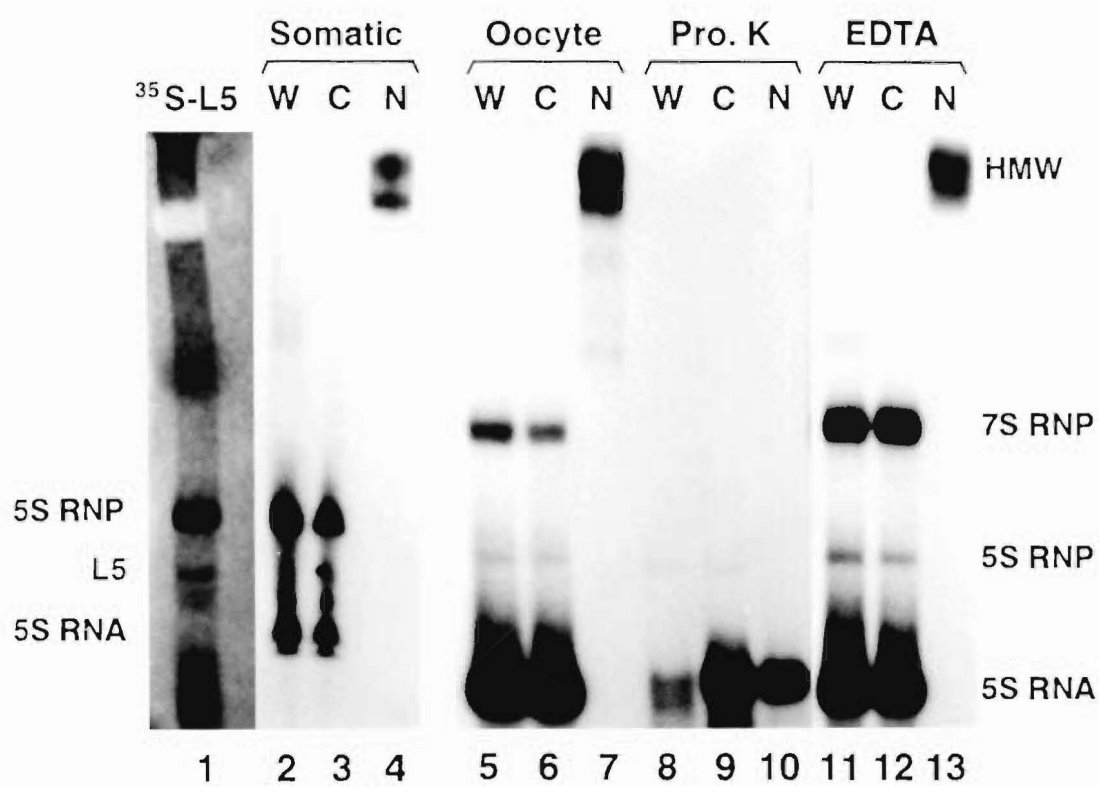
and around nucleoli, although not to the extent shown by somatic-type 5S RNA. Thus, these results support the biochemical nucleolar localisation results for L5, where the amount associated with nucleoli was higher than that observed for oocyte-type 5S RNA, but less than somatic-type.

Since the rabbit reticulocyte lysate contains excess 5S RNA (Zehavi-Willner and Danon, 1972), the majority of L5 synthesised binds to this 5S RNA, forming 5S RNPs (see Section V, Fig. 4-12, lane 1). Also, L5 synthesised using the same method was shown to be bound within 5S RNPs after microinjection into *Xenopus* oocytes (K. J. Murdoch and L. A. Allison, submitted). It is not known, however, whether after microinjection, an exchange reaction takes place, similar to that which can occur *in vitro* (Nazar and Wildeman, 1983; Huber and Wool, 1986b; Allison *et al.*, 1995), whereby the rabbit 5S RNA is replaced by oocyte-type 5S RNA. Thus, the distribution of L5 in the nucleus reflects the distribution of L5 bound to either rabbit reticulocyte 5S RNA or *Xenopus* oocyte-type 5S RNA, or a mixture of the two.

V. 5S RNA-protein Associations in the Nucleus

The above results suggest that there are pools of both 5S RNA and ribosomal protein L5 in the nucleus of oocytes which are not associated with nucleoli. To determine whether these pools of 5S RNA and L5 were associated in the form of 5S RNPs, and to determine if 5S RNA is bound to any other proteins in the nucleus, non-denaturing gel electrophoresis was performed on nuclear homogenates. In contrast to cytoplasmic fractions, where three bands, corresponding to free 5S RNA, 5S RNPs and 7S RNPs have been identified (Fig. 4-12, lanes 5 and 6), nuclear 5S RNA was present in a high molecular weight complex, which could be resolved into two bands (Fig. 4-12, lane 7). This complex was formed by both oocyte-type and somatic-type 5S RNA (compare lanes 4 and 7), as well as many of the mutants tested (data not shown), and was stable in up to 500 mM KCl (data not shown). Treatment of nuclei with proteinase K abolished this complex (lane 10), indicating the involvement of proteins. To determine whether this complex was a preribosomal particle, nuclear homogenates were treated with EDTA, which is known to release

Figure 4-12 Non-denaturing gel electrophoresis of oocyte fractions. Lane 1, *in vitro*-generated ³⁵S-labelled ribosomal protein L5, showing formation of 5S RNPs in the rabbit reticulocyte lysate. Lanes 2 to 13, stage V oocytes were injected with ³²P-labelled somatic-type (lanes 2 to 4) or oocyte-type 5S RNA (lanes 5 to 13) and incubated overnight. Three whole oocytes (W), cytoplasm (C), or ten nuclei (N) were homogenised, and immediately analysed (lanes 2 to 7) or subsequently treated with proteinase K (pro. K) or EDTA. Samples were analysed by 6% PAGE containing 0.1% Triton X-100 followed by autoradiography. HMW, high molecular weight complex containing 5S RNA in nuclei fractions. Lanes 1 to 4 and 5 to 13 represent experiments which were electrophoresed for different lengths of time.



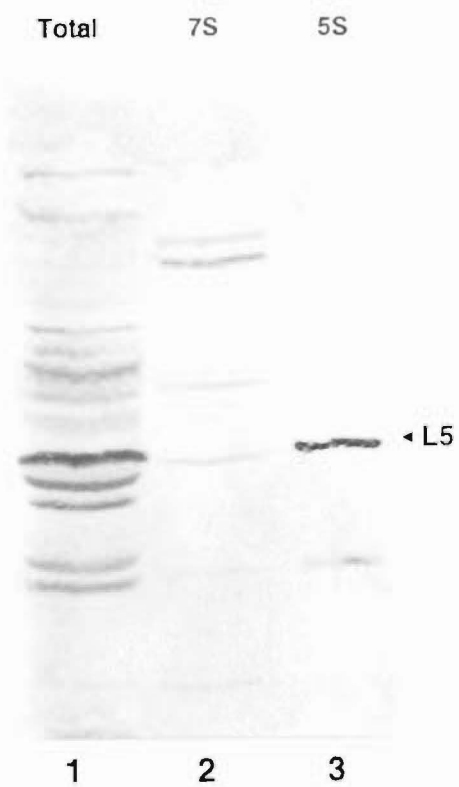
5S RNPs from 60S subunits (Blobel, 1971). EDTA treatment did not release 5S RNA from the complex (Fig. 4-12, lane 13), indicating it is not a precursor ribosomal particle. Although both U1 and U3 snRNAs were shown to form high molecular weight complexes in nuclei under the same conditions, these were not of the same size as that containing 5S RNA (data not shown). The biological significance of this complex was not investigated further, and it is not known whether it represents a real 5S RNA-containing complex found in oocyte nuclei, or is formed by non-specific interactions during preparation of nuclear homogenates.

A second approach was therefore utilised to determine which proteins 5S RNA is associated with in the nucleus. Immunoprecipitation assays were performed using antibodies against the known 5S RNA-binding proteins, TFIIIA and L5, and also 60S ribosomal subunits. Association with another 5S RNA-binding protein, La, was not analysed, since this is thought to bind only transiently to newly transcribed 5S RNA (Gottlieb and Steitz, 1989; Guddat *et al.*, 1990). The anti-TFIIIA and anti-60S ribosomal subunit antisera were described in Chapter 3.

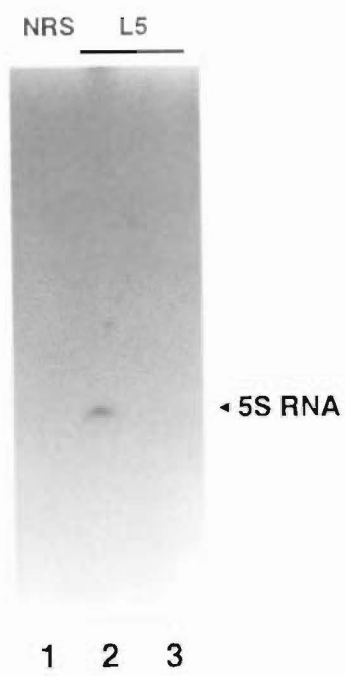
The anti-L5 antibody was prepared against 5S RNPs purified from *Artemia salina*, and specifically recognises L5 in preparations of both *Artemia* and rat 60S subunit proteins (Kenmochi and Ogata, 1989). This anti-L5 antibody also recognised a purified preparation of *Xenopus laevis* L5, and L5 in a sample of total soluble oocyte proteins, as shown by western analysis (Fig. 4-13A, lanes 1 and 3). The antibody did not react above background levels with a preparation of TFIIIA (lane 2), but did show some cross reactivity to other oocyte proteins (lane 1). Secondary reactivities have also been observed with another anti-L5 antibody in HeLa cells (Steitz *et al.*, 1988) and *Xenopus* embryos (Wormington, 1989). To confirm that the anti-L5 antibody was able to recognise *Xenopus* L5 when bound to 5S RNA, oocyte RNAs were labelled with [³²P]GTP and oocyte homogenates immunoprecipitated with the anti-L5 antiserum. The RNA was extracted from immunoprecipitates and analysed by PAGE and autoradiography. Figure 4-13B shows that 5S RNA was the only RNA precipitated with this antibody (lane 2), indicating that 5S RNPs were recognised. The absence of the other rRNAs in immunoprecipitates suggests that intact 60S subunits or 80S ribosomes were not recognised by this antibody. Prior treatment of oocyte extracts with proteinase K

Figure 4-13 Characterisation of *Artemia* anti-L5 antiserum. (A) Total stage V oocyte proteins (Total), purified 7S RNPs (7S) or purified 5S RNPs (5S) were separated by SDS-PAGE and transferred to nitrocellulose membrane. Proteins were visualised by immunoblotting with the anti-L5 antisera. (B) Oocyte RNAs were labelled by injection of ^{32}P [GTP] into the cytoplasm of stage V oocytes followed by an overnight incubation. Oocyte homogenates (lanes 1 and 2), or oocyte homogenates previously treated with proteinase K (lane 3) were immunoprecipitated with normal rabbit serum (NRS) or anti-L5 antisera (L5). RNA was extracted from immunoprecipitates and analysed by 8 M urea/8% PAGE and autoradiography.

A



B



abolished the precipitability of 5S RNA (lane 3), showing that the antibody recognised the protein component of the RNP and not the naked RNA.

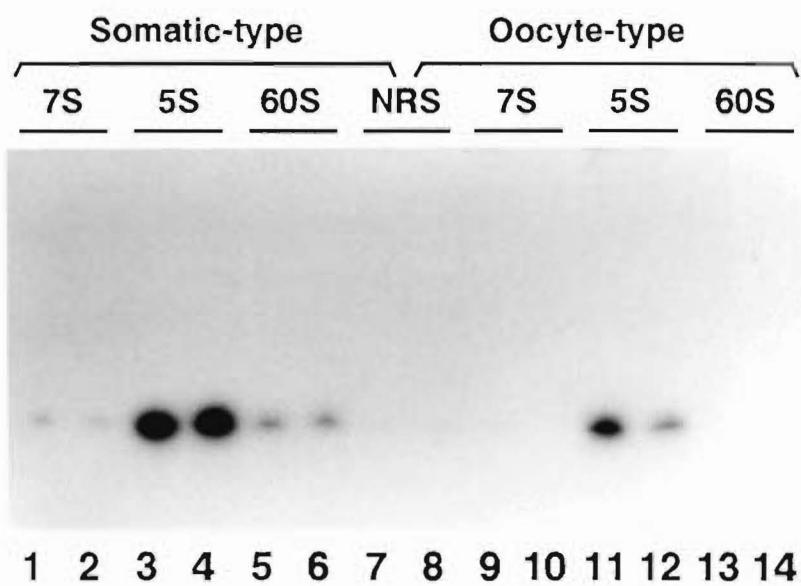
To determine if there were any differences between oocyte-type and somatic-type 5S RNA-protein associations within the nucleus, it was necessary to be able to directly compare immunoprecipitates of the two types of RNA. Previously it was shown that when comparable amounts of RNA were injected into the cytoplasm, twice as much somatic-type 5S RNA was imported into the nucleus compared with oocyte-type (Allison *et al.*, 1995). Thus, to ensure equal amounts were present in the nucleus, twice as much oocyte-type as somatic-type 5S RNA was injected into the cytoplasm of oocytes. After incubation, the nuclei were dissected and subject to immunoprecipitation (Fig. 4-14A). Immunosupernatant fractions show that this treatment resulted in almost identical amounts of each type of 5S RNA present in nuclei fractions (Fig. 4-14B, compare lanes 1-4 and 5-8).

In order to compare amounts precipitated by the different antibodies, it was also important to ensure that saturating quantities of antibody were used. To test this, nuclear homogenates were subject to sequential immunoprecipitations with the same antibody. The sequential immunoprecipitations show that the amounts of the anti-TFIIIA, anti-L5, and anti-60S antibodies used were not saturating, as these complexes were not immuno-depleted during the first precipitation (Fig. 4-14A, cf. lanes 1 and 2, 3 and 4, 5 and 6, 11 and 12). However, subsequent trials with increasing concentrations of anti-L5 antibody failed to increase the amount of 5S RNPs precipitated.

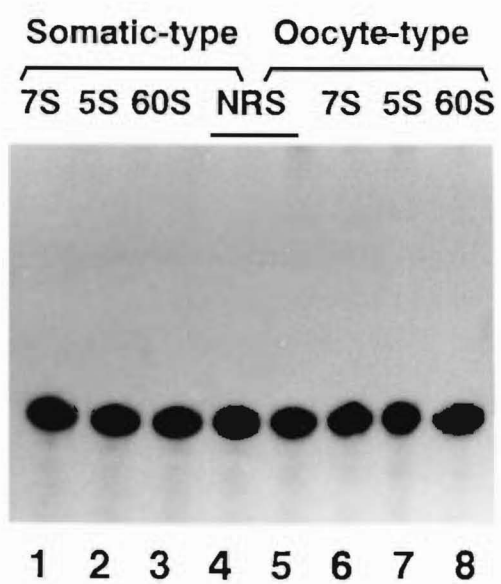
Although these results are not quantitative, both oocyte-type and somatic-type 5S RNAs were clearly predominantly associated with L5 in the nucleus (lanes 3, 4, 11 and 12) with only negligible amounts of 7S RNPs detected (lanes 1, 2, 9 and 10). Since the amounts of antisera used were not saturating, it could be argued that the low amount of 5S RNA immunoprecipitated with the anti-TFIIIA antiserum is because this is a weaker antiserum than the anti-L5 antiserum. However, immunoprecipitations of whole oocytes presented in Chapter 3, which used only half as much anti-TFIIIA antiserum, clearly precipitated greater amounts of labelled 7S RNPs, even though the cytoplasm contains a large store of unlabelled, competing 7S RNPs (see Fig. 3-5A). This suggests that the small amount of 7S RNPs

Figure 4-14 Immunoprecipitation of oocyte nuclear fractions. Stage V oocytes were cytoplasmically injected with ^{32}P -labelled somatic-type or oocyte-type 5S RNA and incubated overnight. Nuclear homogenates from 20 oocytes were immunoprecipitated twice with anti-TFIIIA (7S), anti-L5 (5S), or anti-60S ribosomal subunit (60S) antisera, or with normal rabbit serum (NRS). RNA was extracted from immunoprecipitates after the first and second immunoprecipitations, as shown in consecutive lanes for each antisera (A) or immunosupernatants after the second immunoprecipitation (B), and analysed by 8 M urea/8% PAGE and autoradiography. Immunoprecipitates were exposed for three weeks, immunosupernatants were exposed overnight.

A



B



precipitated from nuclear fractions is, in fact, because there are not many of these RNPs present in this compartment.

The above results are in contrast to the behaviour of oocyte-type 5S RNA in the cytoplasm, where it predominantly associates with TFIIIA (Fig. 4-12, lanes 5 and 6), but supports the proposal that it is L5 that targets 5S RNA to the nucleus for eventual incorporation into the 60S ribosomal subunit. Consistent with the nucleolar localisation results and with previous data (Allison *et al.*, 1995), somatic-type 5S RNA showed greater amounts of ribosome incorporation than oocyte-type (Fig. 4-14, cf. lanes 5 and 6 with lanes 13 and 14). Comparison of oocyte-type and somatic-type 5S RNA reveals that more somatic-type 5S RNA overall was immunoprecipitated with the three antibodies, although there were equal amounts of each RNA in the nucleus (Fig. 4-14A and B). This suggests that a substantial amount of oocyte-type 5S RNA is not associated with TFIIIA or L5, or that, if it is, these proteins are not recognised by their respective antibodies, perhaps due to the binding of some other nuclear component(s).

The above results clearly indicate the presence of 5S RNPs in the nucleus, but do not show whether these RNPs are nucleolar or nucleoplasmic. To determine the intranuclear location of these complexes, immunoprecipitations were performed on nuclear extracts which were depleted of nucleoli using the nucleolar fractionation procedure described above. Since oocyte-type 5S RNA showed lower precipitability in the previous experiment, this assay was performed only with somatic-type 5S RNA. The results clearly show the presence of 5S RNPs in non-nucleolar, nucleoplasmic fractions (Fig. 4-15, lane 1), even though only 30% of somatic-type 5S RNA is found in this fraction (Fig. 4-5). This confirms that at least some of the pool of 5S RNA and L5 in the nucleoplasm is associated in the form of 5S RNPs. No 5S RNA was precipitated with the anti-60S subunit antibody in non-nucleolar fractions, indicating that the majority of the ribosomal subunits are associated with the nucleoli (Fig. 4-15, lane 2).

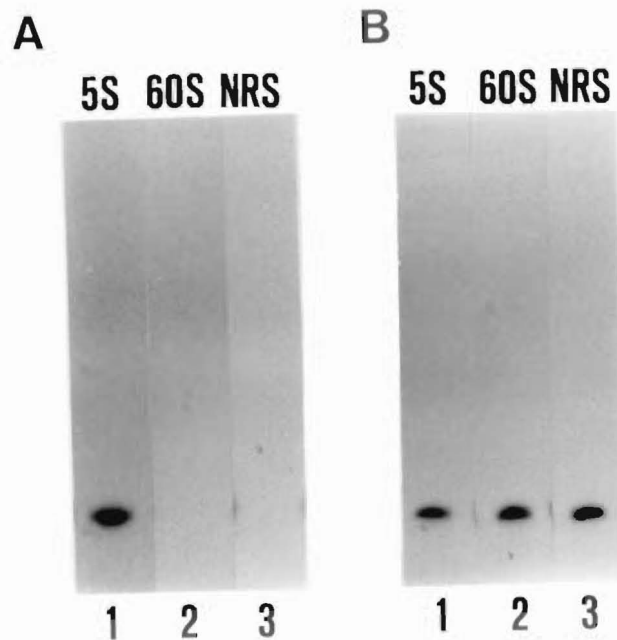


Figure 4-15 Immunoprecipitation of oocyte nucleoplasmic fractions. ^{32}P -labelled somatic-type 5S RNA was injected into the cytoplasm of stage V oocytes, which were then incubated overnight. Nucleoplasmic fractions were prepared from 20 isolated nuclei by sonication and centrifugation, and immunoprecipitated with anti-1.5 (5S) or anti-60S ribosomal subunit (60S) antisera, or with normal rabbit serum (NRS). RNA was extracted from immunoprecipitates (A) and immunosupernatants (B), and analysed by 8 M urea/8% PAGE and autoradiography. Immunoprecipitates were exposed for four weeks and immunosupernatants for three days.

4.3 DISCUSSION

The results presented in this chapter further characterise the distribution of 5S RNA in the nucleus of *Xenopus laevis* oocytes. One of the interesting findings is the low percentage of 5S RNA in the nucleus that is actually associated with nucleoli and therefore being assembled into ribosomal subunits. This was not simply due to an overloading of the system with exogenous RNA, since endogenous 5S RNA showed a similar distribution pattern. Conversely, the low amount of endogenous 5S RNA associated with nucleoli was not simply newly synthesised RNA en route to the nucleolus, since exogenous 5S RNA showed the same nuclear distribution. All of the B23/NO38, which is found in the same nucleolar components as 5S RNA and is also associated with 60S preribosomal particles (Spector *et al.*, 1984; Hügler *et al.*, 1985; Yung *et al.*, 1985; Schmidt-Zachmann *et al.*, 1987; Biggiogera *et al.*, 1989), and all of the 18S and 28S rRNAs were detected in nucleolar fractions. It is therefore unlikely that the low level of 5S RNA associated with nucleoli is due to almost completed ribosomal subunits detaching during the preparation process. The consistency between the endogenous and exogenous 5S RNA results, as well as between the two different techniques utilised, suggests that this pattern reflects the situation *in vivo*. Even in stage IV oocytes, where ribosome assembly is occurring at a maximum rate, only 40% of the endogenous, nuclear 5S RNA was localised to nucleoli. Since oocyte-type 5S RNA is the predominant form of 5S RNA in oocytes, this distribution is likely to reflect that of oocyte-type 5S RNA. These results are consistent with previous *in situ* hybridisation studies which showed that the localisation of 5S RNA over nucleoli peaked in stage IV oocytes (Allison *et al.*, 1991).

I. Requirements for the Nucleolar Localisation of 5S RNA

In contrast to oocyte-type 5S RNA, 70% of somatic-type 5S RNA in the nucleus was associated with nucleoli. This nucleolar localisation of somatic-type 5S RNA resembles that shown by HeLa cell 5S RNA after microinjection into

Xenopus oocytes (De Robertis *et al.*, 1982). Interestingly, somatic-type 5S RNA is more closely related to HeLa cell 5S RNA in sequence than oocyte-type 5S RNA (Ford and Southern, 1973).

The striking difference in nucleolar localisation between oocyte-type and somatic-type 5S RNAs correlates with previous findings on the behaviour of somatic-type 5S RNA in oocytes. Somatic-type 5S RNA was imported into the nucleus at a faster rate and to a greater extent than oocyte-type 5S RNA, and a greater amount was assembled into 60S ribosomal subunits (Allison *et al.*, 1995). These results were explained by the different protein binding activities of the two types of RNA in the cytoplasm: somatic-type was predominantly associated with ribosomal protein L5, whereas oocyte-type preferentially associated with TFIIA, forming storage 7S RNPs (Allison *et al.*, 1995). Since L5 is thought to be involved in the nuclear import of both oocyte-type and somatic-type 5S RNA, all of the 5S RNA in the nucleus would be expected to be associated with L5, rather than TFIIA, unless L5 was replaced by TFIIA in the nucleus. It seems unlikely, therefore, that differences in L5 binding explain the different nucleolar localisation characteristics of the two types of 5S RNA. This is supported by the immunoprecipitation results, which showed that both oocyte and somatic-type were predominantly bound with L5 rather than TFIIA in the nucleus.

Nucleolar localisation studies of L5 showed it to be associated with nucleoli in an amount intermediate to that of oocyte-type and somatic-type 5S RNAs. This L5 is likely to be bound to a mixture of rabbit 5S RNA and oocyte-type 5S RNA. If a higher degree of L5 binding was responsible for the high degree of nucleolar localisation observed for somatic-type 5S RNA, a similarly high proportion of L5 would be expected to localise to nucleoli, irrespective of the type of 5S RNA it was associated with. Since this was not the case, this further supports the notion that the different nucleolar localisation characteristics of oocyte-type and somatic-type 5S RNAs are not conferred by differences in L5 binding. This implies that the sequence differences between oocyte-type and somatic-type 5S RNA, or conformational differences caused by these sequences, are directly recognised by other nuclear factors. These may be other ribosomal and/or nucleolar components, to which somatic-type has a higher affinity, or nucleoplasmic components to which oocyte-type 5S RNA has a higher affinity.

Thus, L5 binding, although hypothesised to mobilise 5S RNA for import into the nucleus in *Xenopus* oocytes, does not appear to determine the distribution of 5S RNA once inside the nucleus. Instead, its own distribution appears to be dependent on the type of 5S RNA to which it is bound. This dependence of L5 on the sequence of 5S RNA to which it is bound for nucleolar localisation could be more directly demonstrated by analysing the distribution of ^{35}S -L5 preassembled with either oocyte-type or somatic-type 5S RNA. This can be achieved by adding excess unlabelled 5S RNA to the L5 *in vitro* transcription-translation reaction, as was previously performed with *Xenopus* L5 (K. J. Murdoch and L. A. Allison, submitted) and yeast L1 (Yeh and Lee, 1995a), to form 5S RNPs.

All of the mutant 5S RNA molecules tested showed some degree of nucleolar localisation. Since mutations in most regions of the molecule were tested, these results imply that a specific sequence of the 5S RNA molecule does not confer nucleolar localisation. This correlates with other studies on nucleolar targeting, where nucleolar localisation is not conferred by a linear sequence, but appears to be dependent on regions of molecules which are involved in binding to other nucleolar components (see Introduction to this chapter).

The series of 5S RNA mutants tested here have also previously been tested for their ability to be imported into the oocyte nucleus after injection into the cytoplasm, where similar results were obtained. All of the mutants were capable of nuclear import, although to varying degrees (Allison *et al.*, 1993; L. A. Allison and P. J. Romaniuk, in prep.). It is interesting that all of the mutants capable of nuclear import and nucleolar localisation are all capable of binding L5 (Chapter 3). It is therefore possible that L5 binding is a necessary, although not sufficient, requirement for the nucleolar localisation of 5S RNA. This would need to be tested using mutant 5S RNA molecules that do not bind to L5. Conversely, the nucleolar localisation of L5 may also require the binding of 5S RNA. This could be tested using mutant L5 molecules which were defective for binding 5S RNA.

II. Nucleoplasmic 5S RNA

The nature of the protein interactions of the 5S RNA in the nucleus not associated with nucleoli was further investigated by immunoprecipitation of nuclear and nucleoplasmic fractions. These assays confirmed that a proportion of this 5S RNA was associated with L5. However, a significant amount of 5S RNA remained in immunosupernatants after immunoprecipitations with either anti-TFIIIA, anti-L5 or anti-60S subunit antisera. This effect was more noticeable for oocyte-type 5S RNA, where less was immunoprecipitated than somatic-type, although more oocyte-type 5S RNA should have been available since less is associated with nucleoli. These results suggest that either 5S RNPs and 7S RNPs are bound to other nuclear components so that they are masked from recognition by their respective antibodies, or that 5S RNA is bound to proteins other than L5 or TFIIIA in the nucleus. The association of more oocyte-type 5S RNA with nucleoplasmic components would also explain the lower levels of nucleolar localisation observed with this RNA. The idea that 5S RNA within the nucleoplasm is bound to other factors is also supported by the results of non-denaturing gel electrophoresis, where all of the nuclear 5S RNA was contained within a high molecular weight complex.

The factors 5S RNA might be associated with in the nucleoplasm were not investigated, but there are several possibilities. These could be factors involved in the nuclear import process. The nuclear pore targeting complex is known to be translocated through the nuclear pore as a single entity. The β -subunit of the complex is released on the nucleoplasmic side of the nuclear envelope, however, importin- α is found distributed throughout the nucleus (Görlich *et al.*, 1995). It is not known at what stage importin- α dissociates from the transport substrate, thus it is possible that the nucleoplasmic 5S RNA is still bound to this protein. Dissociation of this complex may depend on other nuclear factors, which would then allow 5S RNA to be targeted to nucleoli.

Since the GTP-binding protein Ran/TC4 is thought to be important for targeting of newly synthesised U3 snoRNA to nucleoli (Cheng *et al.*, 1995), and is necessary for protein import (reviewed in Moore and Blobel, 1994), this protein may also be associated with nuclear 5S RNA. According to the model of Cheng *et al.* (1995), Ran/TC4 would be released after GTP hydrolysis, catalysed by the presence

of a GTPase-activating protein. In the case of 5S RNPs, the GTPase-activating protein would be located at the nucleolus, resulting in deposition of 5S RNPs at nucleoli. Interestingly, the guanine nucleotide exchange factor, RCC1 was recently shown to form a large complex in *Xenopus* egg extracts, containing Ran/TC4 and three other proteins (Saitoh and Dasso, 1995).

Finally, nucleoplasmic 5S RNA may represent excess RNA in the nucleus, which is being temporarily stored. Thus, the role of the putative 5S RNA-binding factors in the nucleus could be to prevent the efflux of 5S RNA back to the cytoplasm or to regulate the amount of 5S RNA at the nucleolus at one time. Recent studies have suggested that the retention of both proteins and RNAs in the nucleus is due to binding interactions with other nuclear factors (Schmidt-Zachmann *et al.*, 1993; Vancurova *et al.*, 1993; Boelens *et al.*, 1995; Paine *et al.*, 1995; Terns *et al.*, 1995). Retention of excess 5S RNA in the nucleus would ensure continued availability for ribosome synthesis.

III. Nucleolar Localisation of Mutants Defective for Ribosome Assembly

Each of the four mutants which were defective for assembly into 60S ribosomal subunits showed different nucleolar localisation phenotypes. Mutant 10-13 showed a similar nucleolar localisation to oocyte-type 5S RNA. This result indicates that defective nucleolar targeting is not the reason for this mutant not being assembled into ribosomal subunits. Mutant 96-101 showed a lower level of nucleolar accumulation (0.68) relative to oocyte-type. This could reflect either a lower affinity for nucleolar components, or a higher affinity for putative nucleoplasmic 5S RNA-binding components. The lower level of nucleolar localisation of this mutant is probably not the sole reason for it not being detected in 60S subunits, although it may be a contributing factor.

Deletion of the bulged nucleotides at positions 49 and 50 or 63 resulted in 5S RNA molecules defective for ribosome assembly. Both of these mutant RNAs were present in high quantities in nucleolar fractions in the biochemical assay, but did not show a corresponding degree of nucleolar localisation in the *in situ* assays, exhibiting a homogeneous distribution throughout the nucleus. These results indicate

that $\Delta 49,50$ and $\Delta 63$ are binding to other nuclear structures which are large enough to be pelleted under the biochemical fractionation conditions. These larger complexes may result from completely non-specific interactions or may represent some normal, but unknown cellular process. Two other mutant RNAs, one derived from tRNA^{met} and the other from U1 snRNA, have also shown abnormal behaviour in the nucleus of *Xenopus* oocytes. Both of these mutants were defective in nuclear export due to interactions with unidentified, saturable binding sites within the nucleus (Boelens *et al.*, 1995).

The complexes formed by $\Delta 49,50$ and $\Delta 63$ may be part of a degradation pathway for defective 5S RNA molecules. Recently, the Ro antigen was found to be associated with 5S RNA molecules containing point mutations as well as additional nucleotides at the 3' end, suggesting this protein may be part of a 5S RNA discard pathway (O'Brien and Wolin, 1994). However, this seems unlikely here, since of all of the mutants tested, $\Delta 49,50$ and $\Delta 63$ were the only mutants which formed this complex, and these mutants were also of the correct length. In addition, all of the mutants were stable in oocytes during the incubation period, suggesting they were not degraded.

The presence of $\Delta 49,50$ and $\Delta 63$ in nucleolar pellets indicates they are associated with relatively large or insoluble complexes in the nucleus. One such component of the nucleus is the nuclear matrix. This term refers to the elaborate three-dimensional proteinaceous structure which remains after high salt extraction and removal of DNA from nuclei. The nuclear matrix is thought to be the site of DNA replication, heterogeneous RNA (hnRNA) processing and steroid hormone action, and to also determine the higher order chromatin architecture (reviewed in Berezney, 1991). Preparations of nuclear matrix fractions from oocyte nuclei showed that both oocyte-type 5S RNA and $\Delta 49,50$ were contained in the soluble, supernatant fraction (pers. observ.), ruling out the possibility that association with the nuclear matrix is the reason for these mutants' aberrant behaviour.

Other large structures within *Xenopus* oocyte nuclei include different types of granules (referred to as spheres, sphere organelles or snurposomes), which range from one to ten microns in diameter. These have been shown to contain various components of mRNA splicing machinery, as well as a homologue of p80-coilin, suggesting they are related to the coiled bodies found in plant and animal cells (Wu

et al., 1991; Tuma *et al.*, 1993; Wu and Gall, 1993). Since p80-coilin may perform a structural role in the nucleolus (Bohmann *et al.*, 1995) and coiled bodies are known to contain nucleolar components such as fibrillarin (Raška *et al.*, 1991), U3 snoRNA (Azum-Gélade *et al.*, 1994; Jiménez-García *et al.*, 1994), Nopp140 and its binding protein NAP57 (Meier and Blobel, 1994) and ribosomal protein S6 (Jiménez-García *et al.*, 1994), and have been identified within the nucleoli of certain breast cancer cells (Ochs *et al.*, 1994) and in hepatocyte nuclei from hibernating dormice (Malatesta *et al.*, 1994), it is possible that the granules present in amphibian oocyte nuclei may also contain nucleolar components. 5S RNPs were not identified in coiled bodies in mammalian cells (Raška *et al.*, 1991), however, examination of their presence in amphibian oocyte granules has not been reported. Thus, it is possible that 5S RNA or the mutants $\Delta 49,50$ and $\Delta 63$ are associated with these granules in the nuclei of oocytes.

Recent evidence suggests that bulged nucleotides are capable of modulating the strength of protein-RNA interactions. For example, the *E. coli* ribosomal protein S8 binds to both 16S rRNA and to its own mRNA transcript, acting as a repressor of translation (reviewed in Nomura *et al.*, 1984). The S8 binding site on 16S rRNA consists of two short duplexes that enclose a conserved, asymmetric internal loop. The S8 binding site on the mRNA is very similar, in both primary and secondary structure, except for the presence of two single bulged nucleotides (Wu *et al.*, 1994). The two bulged nucleotides were shown to confer a five-fold decrease in the binding affinity of S8 for the mRNA, where deletion of the bulged nucleotides increased S8 binding five-fold (Wu *et al.*, 1994).

It is tempting to speculate therefore, that deletion of the bulged nucleotides in 5S RNA has a similar effect. The resulting 5S RNA would have a higher affinity for nucleoplasmic components, which may even be part of the normal pathway taken by 5S RNA within the nucleus. The mutant RNAs would thus remain associated with these components, possibly forming aggregates, resulting in complexes large enough to be pelleted during the centrifugation step. This hypothesis is supported by the fact that $\Delta 49,50$ and $\Delta 63$, both of which contain deletions of bulged nucleotides, were the only mutants showing this aberrant phenotype, with the majority of the other mutants showing similar distribution characteristics to oocyte-type.

Whatever the composition of these large complexes, it is possible that their formation prevents these mutant 5S RNAs being integrated into ribosomal subunits. This provides an alternative explanation to that proposed in Chapter 3, where deletion of the bulged nucleotides was postulated to prevent incorporation into 60S subunits due to a loss of binding interactions with other ribosomal components. Further studies are necessary to distinguish between these two possibilities.

IV. Pathway of 5S RNA to the Ribosome

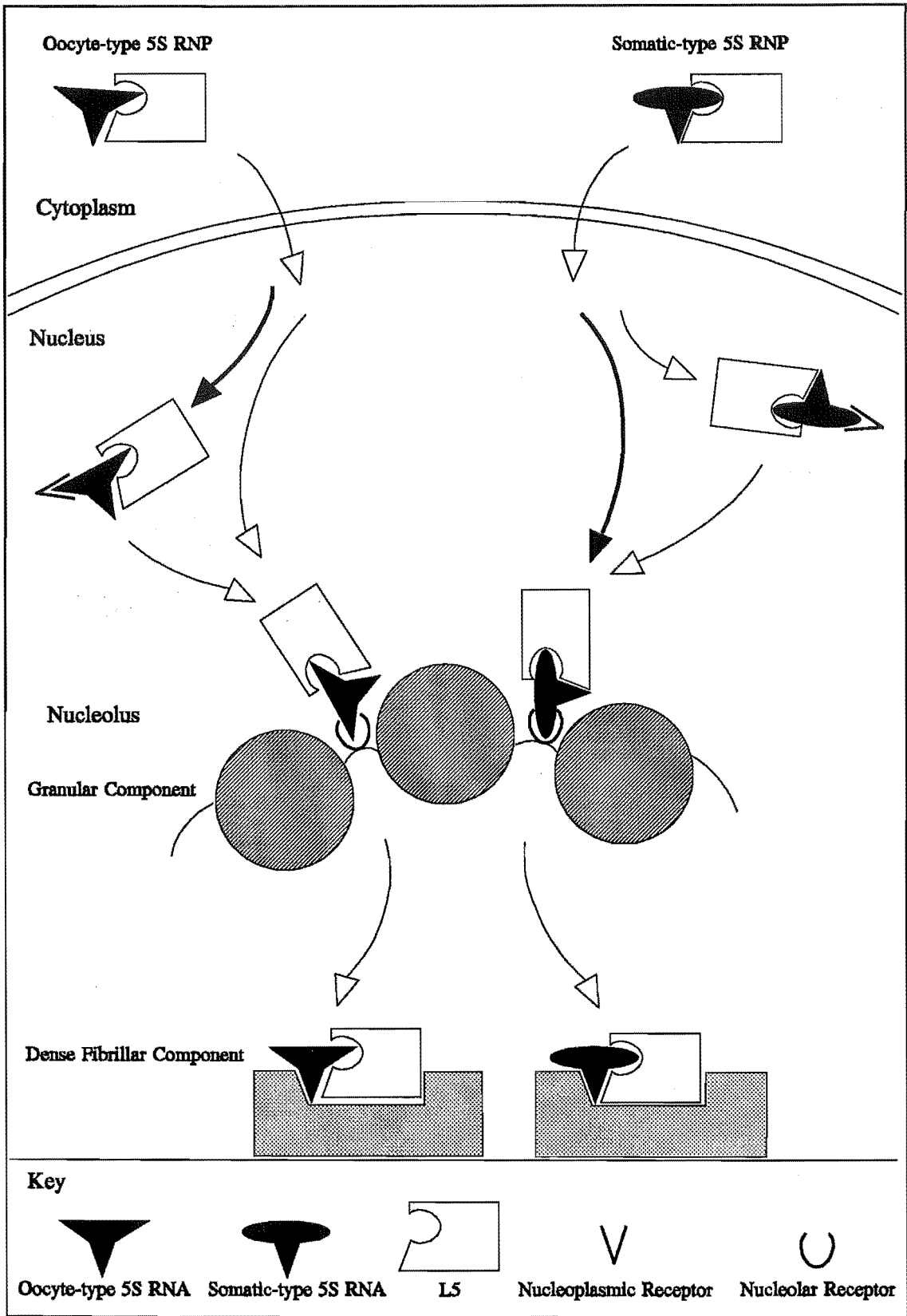
As discussed in the Introduction to this chapter, 5S RNA is thought to be assembled into eukaryotic ribosomal subunits at a relatively early stage of ribosome assembly, in the dense fibrillar component of the nucleolus (Knight and Darnell, 1967; Warner and Soeiro, 1967; Raška *et al.*, 1995; Shaw *et al.*, 1995). As these sites might not be readily accessible from the outside of the nucleolus, it could be proposed that 5S RNA binds to other nucleolar components, which act as receptors and provide a pathway to the site of integration into preribosomal particles. Since approximately equal amounts of oocyte-type and somatic-type 5S RNAs were injected into oocytes in the nucleolar localisation studies, yet more than twice as much somatic-type was associated with nucleoli compared with oocyte-type and endogenous 5S RNA, this implies that more molecules of somatic-type 5S RNA are associated with nucleoli. This suggests that under *in vivo* conditions, not all possible 5S RNA binding sites within the nucleolus are occupied. If 5S RNA binds directly to preribosomal particles within the nucleolus, this would imply that 5S RNA is a limiting factor during ribosome assembly. This seems unlikely in view of the large amount of 5S RNA stored in the cytoplasm and, as shown here, present in the nucleoplasm. The alternative scenario is that there are additional 5S RNA binding sites at the nucleolus before the molecule becomes assembled into preribosomal particles. If somatic-type 5S RNA has a higher affinity for these sites, this would explain the higher levels of nucleolar accumulation observed for this RNA. Thus, the data presented here support the above hypothesis, whereby 5S RNA interacts with other nucleolar components before being integrated into preribosomal particles.

Figure 4-16 depicts a model which proposes the pathway taken by 5S RNA within the nucleus, incorporating the differences observed between oocyte-type and somatic-type 5S RNAs. As shown in this model, oocyte-type and somatic-type 5S RNAs have slightly different conformational structures resulting from the six nucleotide substitutions. This confers different binding affinities on the two types of RNA for different oocyte proteins. Once imported into the nucleus bound to ribosomal protein L5, oocyte-type 5S RNA-containing 5S RNPs bind to nucleoplasmic components, to which they have a high affinity. Thus, only a small percentage of oocyte-type 5S RNA is associated with nucleoli. Somatic-type 5S RNA-containing 5S RNPs, however, have a lower affinity for these nucleoplasmic factors, but a high affinity for nucleolar receptors. Thus, more somatic-type 5S RNA in the nucleus is associated with nucleoli and more is assembled into 60S ribosomal subunits.

In this model, therefore, the intranuclear distribution of 5S RNPs is dependent on the sequence of the 5S RNA, rather than L5. Incorporation into preribosomal particles, however, requires sequences and/or structures from both the RNA and protein moieties of the 5S RNP.

The identification of both nucleolar and nucleoplasmic factors which interact with 5S RNA, and the involvement of known nucleolar proteins such as B23 and nucleolin remains to be determined. The model proposed here provides a framework for further experimentation which may provide insights into the enigmatic way in which ribosomes are assembled in the nucleoli of eukaryotic cells.

Figure 4-16 Model for the pathway taken by oocyte-type and somatic-type 5S RNAs in *Xenopus* oocyte nuclei. Oocyte-type and somatic-type 5S RNAs are shown to have a slightly different conformational structure, which alters their relative binding affinities for oocyte proteins. In the cytoplasm, 5S RNA binds to ribosomal protein L5, to which somatic-type 5S RNA has a higher affinity (Q. You, Q. Zang, P. J. Romaniuk, in prep.), and the resulting complex is transported into the oocyte nucleus. In the nucleus, oocyte-type 5S RNA-containing 5S RNPs are proposed to have a higher affinity for nucleoplasmic components (nucleoplasmic receptor), so are more likely to bind these factors (indicated by thick arrows) than to be targeted to nucleoli. Somatic-type 5S RNA is shown to have a higher affinity for receptor molecules on the outside of the granular component of the nucleolus (indicated by thick arrows). After binding to these receptors 5S RNPs make their way to the dense fibrillar component of the nucleolus, where structures of both the RNA and protein are necessary for integration into preribosomal particles.



CHAPTER 5

7S RNP LOCALISATION IN THE CYTOPLASM

5.1 INTRODUCTION

After 5S RNA is synthesised in the nucleus of previtellogenic oocytes, it is exported to the cytoplasm where it is stored in one of two RNA-protein complexes, which sediment at 42S and 7S. The 42S RNP consists of 5S RNA, tRNA and two non-ribosomal proteins (Ford, 1971; Picard *et al.*, 1980). The remaining fifty percent of 5S RNA is complexed in a one to one ratio with the protein TFIIA, as a 7S RNP (Picard and Wegnez, 1979; Honda and Roeder, 1980; Pelham and Brown, 1980). Immunocytochemical studies performed on immature and mature oocytes showed that 42S RNPs and 7S RNPs are located exclusively in the cytoplasm (Mattaj *et al.*, 1983; Viel *et al.*, 1990). In addition, immunoprecipitations of cytoplasmic versus nuclear fractions from oocytes injected with labelled 5S RNA showed that the majority of 7S RNPs formed were present in the cytoplasm, whereas 5S RNPs were found in both compartments (Allison *et al.*, 1991; 1993; Chapter 4). Since TFIIA is also a transcription factor which therefore functions in the nucleus (Engelke *et al.*, 1980), it appears that binding to 5S RNA alters the subcellular location of this protein.

The combined size of TFIIA (38.5 kD; Ginsberg *et al.*, 1984) and 5S RNA (40 kD) is 78.5 kD, which is above the estimated limit for passive diffusion of

globular proteins through nuclear pores (Bonner, 1975; Peters, 1984). Thus, one explanation for the observed cytoplasmic localisation of 7S RNPs is that they are too large to diffuse into the nucleus. However, this seems an unlikely explanation, since 7S RNPs are thought to form in the nucleus and subsequently move through the nuclear pores into the cytoplasm (Guddat *et al.*, 1990). Another possibility for the cytoplasmic location of 7S RNPs is that 5S RNA binding masks the putative TFIIA nuclear localisation sequence (NLS), which has been speculated to lie between the DNA binding domain and transcriptional activation domain (Mao and Darby, 1993).

Studies performed by L. A. Allison (unpubl. observ.) using somatic cell nuclei cultured in a *Xenopus* egg extract showed that both fluorescently labelled TFIIA and 7S RNPs were imported into these nuclei, under conditions where non-nuclear proteins were excluded. These data suggest that the cytoplasmic location of these molecules in oocytes is not due to masking of the TFIIA NLS or due to the large size of the molecule. This implies that another cellular factor(s) is responsible for retaining 7S RNPs in the cytoplasm of oocytes. Such a factor(s) could be some component of the cytoplasm that binds to 7S RNPs, and thus sequesters them, or some component of the nuclear transport machinery that is necessary for 7S RNP import and was present in the *in vitro* extract above, but is absent in oocytes. The latter possibility has some experimental support from studies performed by Featherstone *et al.* (1988). These authors showed that of a group of antibodies which recognise nuclear pore complex proteins in rat liver and kidney cells and *Xenopus* A6 kidney cells, only one of these (RL1) reacted with *Xenopus* oocytes, suggesting that there is cell-specific expression of nuclear pore epitopes. However, the absence of nuclear import factors also seems an unlikely explanation for the cytoplasmic retention of 7S RNPs for the following two reasons. Firstly, TFIIA is capable of nuclear import in oocytes, so necessary import factors must be present for the nuclear import of this molecule. Secondly, although a small amount of 5S RNA has been detected bound to a TFIIA-like protein in HeLa cells (Lagaye *et al.*, 1988), 7S RNPs are not a predominant form of 5S RNA-containing complex, as in oocytes, so it would be unlikely that somatic cells would contain specific nuclear import factors for 7S RNPs.

It was therefore of interest to determine if any oocyte cytoplasmic components play a role in retaining 7S RNPs in the cytoplasm. Such components

could include insoluble filamentous structures, such as the cytoskeleton and internal membrane systems. Experimental evidence for the role of each of these components in the regulation of nuclear import and/or subcellular localisation is reviewed in the following sections.

I. Evidence for the Role of the Cytoskeleton in Localising Proteins and RNA

The cytoskeleton of eukaryotic cells contains three major classes of fibres: the seven nanometre diameter microfilaments, 24 nanometre diameter microtubules and the ten nanometre diameter intermediate filaments. Microfilaments are formed by the polymerisation of the protein subunit G-actin. They also contain a diverse array of other actin binding proteins, which enable the filaments to form unique structures, or to carry out specific cellular functions (reviewed in Schliwa, 1986). Microtubules are formed by the polymerisation of tubulin subunits, and are involved in mitotic spindle formation, chromosome movement and cell separation during mitosis, movement of cilia and flagella, and provide tracks for the transport of small vesicles within the cytoplasm. Intermediate filaments are also made up of subunit proteins, of which there are five major classes; vimentin, desmin, glial fibrillary acidic protein, the neurofilaments and the cytokeratins; each of which is characteristically expressed in a single cell type (reviewed in Schliwa, 1986). The three cytoskeletal components form a complex filamentous system which extends throughout the cytoplasm of cells and is responsible for maintaining cell shape and rigidity, cell motility, and organelle movement and localisation. Other potential functions of the cytoskeleton appear to be in retention and localisation of proteins and RNAs in the cytoplasm.

i. Protein retention

An example of a protein which is retained in the cytoplasm due to associations with the cytoskeleton is the glucocorticoid receptor. The glucocorticoid receptor interacts with specific DNA sequences involved in the regulation of gene transcription. In the absence of glucocorticoid hormone, the receptor is localised to the cytoplasm, but upon addition of hormone it is rapidly translocated to the nucleus.

Immunofluorescence microscopy showed that the receptor is localised in the cytoplasm in a similar pattern to tubulin, in both interphase and mitotic cells (Wikström *et al.*, 1987; Akner *et al.*, 1990). In addition, treatment of cells with colchicine or vinblastine, which depolymerise microtubules, caused a similar redistribution of both glucocorticoid receptor and tubulin to the cell periphery (Akner *et al.*, 1990). In the cytoplasm, the receptor is bound in a multiprotein complex which includes a dimer of heat shock protein 90 (hsp90; reviewed in Pratt, 1992). hsp90 has also been colocalised with microtubules in a variety of cell lines (Redmond *et al.*, 1989). It has thus been proposed that the glucocorticoid receptor is docked in the cytoplasm, in association with the cytoskeleton (Pratt, 1992). Addition of hormone would result in the dissociation of the receptor from hsp90, thus releasing it from its cytoplasmic anchoring site and allowing it to be targeted to the nucleus (Pratt, 1992).

ii. RNA association with the cytoskeleton

a. Somatic cells

Lenk *et al.* (1977), using Triton X-100 extraction, first showed that mRNA was associated with the cytoskeleton of HeLa cells. Extraction of cells with this non-ionic detergent solubilises cellular lipids and membranes, and removes mitochondria, endoplasmic reticulum and 75% of cytoplasmic proteins. The structure of the remaining cytoskeleton, as judged by electron microscopy, was shown to resemble that of intact cells (Lenk *et al.*, 1977). These workers observed that most of the polyribosomes remained associated with the cytoskeleton after detergent extraction. Degradation of mRNA with low levels of ribonuclease released the ribosomes from the cytoskeleton, and disaggregation of polyribosomes with fluoride ions resulted in the release of ribosomes but not the mRNA, suggesting polyribosomes were attached to the cytoskeleton via mRNA (Lenk *et al.*, 1977; Bag and Pramanik, 1987; Taneja *et al.*, 1992).

Since these initial findings, most studies on RNA-cytoskeletal interactions have focussed on mRNAs which are localised within specific regions of the

cytoplasm. Many studies using *in situ* hybridisation have shown specific mRNAs are localised to different regions within somatic cells (Lawrence and Singer, 1986; Garner *et al.*, 1988; Cripe *et al.*, 1993; Kislauskis *et al.*, 1993; Hesketh *et al.*, 1994; reviewed in Bassel, 1993). For example, in chicken embryonic myoblasts and fibroblasts, mRNA encoding actin was found to be predominantly localised to the periphery of cells in lamellipodia, vimentin mRNA was distributed near the nucleus and tubulin mRNA appeared most concentrated in the peripheral cytoplasm (Lawrence and Singer, 1986).

Visualisation of individual mRNA molecules simultaneously with cytoskeletal filaments in human fibroblasts showed that 72% of poly(A) mRNA was localised within five nanometres of orthogonal networks of actin filaments (Singer *et al.*, 1989; Bassel *et al.*, 1994). Poly(A) mRNA also colocalised with vimentin filaments (29%) and microtubules (less than ten percent; Bassel *et al.*, 1994). In addition, treatment of cells with cytochalasins, causing a depolymerisation of actin and collapse of the microfilament network has been shown to release mRNA (Lenk *et al.*, 1977; Bird and Sells, 1986; Ornelles *et al.*, 1986; Taneja *et al.*, 1992) and polysomes (Vedeler *et al.*, 1991) from the cytoskeletal fraction. Thus, the majority of evidence indicates that the cytoskeletal component to which mRNA is bound in somatic cells is the microfilaments.

However, there is also evidence for mRNA association with other cytoskeletal components. Localisation studies of the mRNA 5'-cap binding protein in baby hamster kidney cells showed it to be present in a fibrous network extending throughout the cytoplasm in a radial arrangement, in a pattern directly superimposable on the labelling patterns shown by intermediate filaments (Zumbe *et al.*, 1982). The mRNA cap binding protein was also associated with the cytoskeletal fraction after Triton X-100 extraction and behaved like intermediate filaments in colchicine treated cells, suggesting a direct or indirect linkage with intermediate filaments (Zumbe *et al.*, 1982). In addition, synthesis of viral proteins in cells infected with frog virus 3 is thought to occur on intermediate filaments (Murti *et al.*, 1989).

There is also evidence for mRNA association with microtubules in somatic cells. Visualisation of the subcellular distribution of mRNA encoding myelin basic protein after injection into oligodendrocytes showed that the mRNA formed granules,

which were aligned in tracks along microtubules traversing the cytoplasm and processes (Ainger *et al.*, 1993). Extraction of cells with Triton X-100 did not release these mRNA granules, indicating an association with the cytoskeleton. Also, studies in primary neuronal cells have shown that tau mRNA, which is localised in the cell body and to the proximal portion of the axon, is associated with the microtubule system and not with the actin filaments (Litman *et al.*, 1994). It has been suggested that microtubules function in the transport of mRNA within cells (reviewed in Suprenant, 1993).

b. *Drosophila* oocytes

Specific subcellular localisation of mRNAs is not restricted to somatic cells, and is now known to be an important requirement in eggs for the establishment of protein gradients that give rise to the embryonic body plan (reviewed in St Johnston, 1995). In *Drosophila* oocytes, localisation of a set of mRNAs including *bicoid* (Berleth *et al.*, 1988), *oskar* (Kim-Ha *et al.*, 1991), *nanos* (Wang and Lehmann, 1991), *fs(1)K10* (Cheung *et al.*, 1992) and *orb* (Lantz and Schedl, 1994) is responsible for the formation of both antero-posterior and dorso-ventral axes during embryogenesis.

Most evidence points towards the role of microtubules in transport and localisation of these mRNAs. For example, *bicoid* mRNA is transcribed in the nurse cells and transported into the oocyte via the ring canals, where it is localised to the anterior cortex (Berleth *et al.*, 1988). Drugs that depolymerise microtubules perturb all aspects of this localisation, whereas cytochalasin has no effect (Pokrywka and Stephenson, 1991; Theurkauf *et al.*, 1993). In addition, cellular fractionation using Triton X-100 extraction showed that *bicoid*, *Bicaudal D*, *fs(1)K10*, *orb* and *oskar* mRNAs were all recovered in the cytoskeletal fraction and were released into the soluble fraction after treatment with colchicine, also indicating a role for microtubules (Pokrywka and Stephenson, 1994). However, it appears factors other than microtubules may be required to maintain localisation (Pokrywka and Stephenson, 1995).

c. *Xenopus* oocytes

There are also examples of mRNAs associated with the cytoskeleton in *Xenopus* oocytes. As above, these studies have focussed on RNAs which show localisation to specific regions of the cytoplasm. However, there is no reason to believe RNA molecules that are distributed homogeneously throughout the cytoplasm, such as 7S RNPs, could not also be associated with the cytoskeleton. In *Xenopus* oocytes many mRNAs have been identified and cloned which have a non-homogeneous distribution relative to the animal-vegetal axis (King and Barklis, 1985; Rebagliati *et al.*, 1985; Mosquera *et al.*, 1993). mRNAs localised to the vegetal hemisphere include Vg1 (Rebagliati *et al.*, 1985), which encodes a protein which is a member of the TGF β family of proteins and is thought to function in the generation of dorsal mesoderm (Weeks and Melton, 1987a; Thomsen and Melton, 1993); Xcat2, which has homology to the *Drosophila nanos* protein and is possibly an RNA-binding protein; Xcat3 (Mosquera *et al.*, 1993); and *Xwnt-11*, whose protein is also thought to be important for dorso-ventral axis formation (Ku and Melton, 1993). mRNAs localised to the animal hemisphere include An1 (Rebagliati *et al.*, 1985), which encodes a protein with homology to ubiquitin and contains a putative zinc-finger motif (Linnen *et al.*, 1993); An2 (Rebagliati *et al.*, 1985), which codes for a subunit of mitochondrial ATPase (Weeks and Melton, 1987b); An3 (Rebagliati *et al.*, 1985), whose protein has an ATP-dependent RNA helicase activity (Gururajan *et al.*, 1994); and xlan4 which may have a neural-specific function (Reddy *et al.*, 1992).

Extraction of stage VI *Xenopus* oocytes with Triton X-100 showed that only small amounts of total RNA (0.5%-2%) or poly(A) RNA (0.5%-5%; depending on the extraction procedure used) were present in the detergent insoluble, cytoskeletal fraction (Pondel and King, 1988; Yisraeli *et al.*, 1990). Many of the localised mRNAs in *Xenopus* oocytes are enriched in these cytoskeletal fractions, for example Vg1 (Pondel and King, 1988; Yisraeli *et al.*, 1990; Forristall *et al.*, 1995), Xcat2 and Xcat3 (Mosquera *et al.*, 1993; Forristall *et al.*, 1995). The best studied localised mRNA in *Xenopus* oocytes is Vg1. In young, stage I and II oocytes, Vg1 mRNA is uniformly distributed throughout the cytoplasm. During stage III, Vg1 begins to localise to a tight cortical shell (Melton, 1987). Following hormonal maturation of the oocyte into an egg, Vg1 is released from the tight cortical shell and forms a broader band in the vegetal hemisphere which remains in vegetal cells throughout

early embryogenesis (Weeks and Melton, 1987a). Localisation of Vg1 mRNA appears to be a two step process. The use of cytoskeletal inhibitors suggested that microtubules were involved in the translocation of the message to the vegetal hemisphere, and microfilaments were important for the anchoring of the message at the cortex (Yisraeli *et al.*, 1990). In addition, a proportion of Vg1 mRNA was shown to be associated with microtubules *in vivo* (Elisha *et al.*, 1995). There is also some evidence that intermediate filaments may be involved in anchoring Vg1 mRNA at the vegetal cortex (Pondel and King, 1988; but see also Klymkowsky *et al.*, 1991). Thus, as the above data indicates, there is evidence for a role of all three major cytoskeletal components in the localisation of mRNA within cells.

mRNAs are not the only subset of RNA thought to be associated with the cytoskeleton in *Xenopus*. Xlsirts are a family of interspersed repeat RNAs from *Xenopus* that contain from three to thirteen repeat units (each 79-81 nucleotides long) flanked by unique sequences, that do not appear to be translated (Kloc *et al.*, 1993). Xlsirt RNA is first associated with the mitochondrial mass in stage II oocytes, and is translocated to the vegetal cortex at early stage III (Kloc *et al.*, 1993). Xlsirt repeat sequences are required for this translocation, and can cause the translocation of heterologous RNA (Kloc *et al.*, 1993). Treatment of stage VI oocytes with cytochalasin partially released Xlsirt RNA from the vegetal cortex, indicating that anchoring at the vegetal cortex is partly dependent on microfilaments (Kloc and Etkin, 1995). Interestingly, when endogenous Xlsirt RNA was destroyed using antisense oligodeoxynucleotides, Vg1 mRNA, but not Xcat2 mRNA was released from the vegetal pole, indicating that Xlsirt RNAs are important for Vg1 localisation (Kloc and Etkin, 1994).

d. Requirements for localisation

Experiments using protein synthesis inhibitors such as puromycin and cycloheximide showed that neither the translocation or anchoring of actin mRNA was affected by these inhibitors in chicken embryo fibroblasts (Sundell and Singer, 1990). In *Xenopus*, *in vitro* synthesised Vg1 mRNA microinjected into oocytes localised in a similar fashion to endogenous Vg1 transcripts. Molecules with deletions in the 5' region, which abolished ribosome binding and translation, were also correctly

localised, showing that the necessary information for localisation is present in the naked RNA molecule itself (Yisraeli and Melton, 1988).

Studies using deletion mutagenesis and chimeric mRNA molecules have found that for all mRNAs examined, the 3' untranslated region (3' UTR) of the RNA molecule is both necessary and sufficient for mRNA localisation, in somatic cells, oocytes and embryos (Macdonald and Struhl, 1988; Davis and Ish-Horowicz, 1991; Cheung *et al.*, 1992; Dalby and Glover, 1992; Gavis and Lehmann, 1992; Kim-Ha *et al.*, 1993; Macdonald *et al.*, 1993; Kislauskis *et al.*, 1994; Lantz and Schedl, 1994). For example, in *Xenopus*, a 340 nucleotide region of the 3' UTR is necessary to localise microinjected Vg1 mRNA, and is also capable of localising a chimeric, non-localised mRNA (Mowry and Melton, 1992). Most analyses have found very little homology among the 3' UTRs of localised mRNAs, and it has been suggested that it is the secondary structure which may be recognised by cellular factors (Macdonald and Struhl, 1988; Macdonald, 1990).

The elucidation of the *trans*-acting cellular factors involved in binding to localised mRNA molecules is less well characterised. Proteins have been identified from the cytoskeletal compartment of NIH 3T3 cells and from mouse spermatids that bind to the 3' UTR of localised mRNAs, and have been suggested to provide a link between mRNAs and the cytoskeleton (Sharpless *et al.*, 1993; Schumacher *et al.*, 1995). In *Drosophila* oocytes, localisation of *bicoid* involves at least three steps, each with different protein requirements (for example, Ferrandon *et al.*, 1994; reviewed in St Johnston, 1995). In *Xenopus*, a 69 kD protein has been identified which binds to the Vg1 3' UTR. This binding was inhibited by another vegetally localised mRNA, TGF β 5, but not by the animal pole localised An2 mRNA (Schwartz *et al.*, 1992). This protein was also shown to associate with microtubules, and is required for the specific association of Vg1 mRNA with microtubules *in vitro*, thus is likely to play a role in the localisation of Vg1 mRNA in oocytes (Elisha *et al.*, 1995).

iii. RNP associations with the cytoskeleton

In addition to RNA, there is evidence that different types of RNPs are associated with the cytoskeleton. One example is prosomes, a class of ubiquitous RNPs which consist of variable sets of proteins and RNA, depending on the cell

type, which were first discovered as subcomplexes of translationally repressed mRNP (reviewed in Scherrer, 1990). Fractionation with Triton X-100 showed a significant proportion of prosome protein was associated with the cytoskeletal fraction, and immunofluorescence and immunoelectron microscopy showed that prosome antigens are extensively associated with the cytokeratin-type intermediate filaments in HeLa cells and rat kangaroo kidney epithelial cells (Grossi de Sa *et al.*, 1988). In human fibroblasts, which have an intermediate filament network exclusively constituted of vimentin, prosomes coincide fully with vimentin fibres; and in proliferating mouse myoblasts, where desmins are the dominant constituent of intermediate filaments, the prosomal antigens were found partially located on the desmin network (Olink-Coux *et al.*, 1994). Prosoemes have also been detected in *Xenopus* oocytes and eggs (Ryabova *et al.*, 1994). In previtellogenic oocytes, prosomal antigens were clustered throughout the cytoplasm and nucleus. During vitellogenesis, prosomal proteins acquired a preferential subcortical localisation; and in the fully grown oocyte, staining was more intense in the animal hemisphere, as well as in cortical and subcortical regions. Double staining of stage VI oocytes demonstrated a correlation in the localisation of actin and myosin and prosome particles (Ryabova *et al.*, 1994). Thus, it appears that in different cell types prosomes are associated with different components of the cytoskeleton.

Large RNPs called vaults, which have been described in mammals, amphibians, birds, and the lower eukaryote *Dictyostelium*, consist of multiple copies of a small RNA and more than 50 copies of a single polypeptide (reviewed in Kedersha and Rome, 1990). In stationary fibroblasts, a subpopulation of vaults cluster at the ends of actin stress fibres, while in spreading fibroblasts vaults appear clustered within the actin-rich ruffling edges of lamellipodia (Kedersha and Rome, 1990).

Subcellular fractionation of HeLa S3 cells showed that a group of small cytoplasmic RNAs (scRNAs) were present in the cytoskeletal, non-polyribosome associated fraction, and that a significant proportion of these scRNAs were released by treatment with cytochalasin (Bird and Sells, 1986). These workers also demonstrated the presence of 5S RNA in all fractions analysed, including the cytoskeletal, non-polyribosome associated fraction. This indicates that in HeLa cells a proportion of 5S RNA which is not contained within polyribosomes is associated

with the Triton X-100 cytoskeletal fraction. It is not known what proteins were bound to the 5S RNA present in this fraction.

II. Evidence for the Role of Intracellular Membranes in Localising Proteins and RNA

As well as the plasma membrane, eukaryotic cells contain an extensive system of internal membranes, which enclose and separate specific regions from the rest of the cytoplasm. The largest component is the endoplasmic reticulum, a network of interconnected closed membrane vesicles in which protein and lipids are synthesised. Membranes also form stacks of flattened sacs which constitute the Golgi apparatus, which is involved in transporting membrane constituents to appropriate places within the cell. Membranes also surround the nucleus, mitochondria, and smaller organelles such as lysosomes and peroxisomes (reviewed in Evans and Graham, 1989). Another membranous component of cells is the annulate lamellae, which consist of parallel membranes, often stacked, that enclose a cavity, or cisterna, and contain numerous pore complexes which are structurally similar to those of the nuclear envelope (reviewed in Kessel, 1992). Although the function of annulate lamellae is unknown, there have been many proposed functions, including a reservoir of nuclear envelope components, biogenesis of mitochondria, steroid production, and roles in the release, packaging or assembly of stored developmental information, such as mRNA (reviewed in Kessel, 1992).

i. Protein retention

Type II cyclic AMP (cAMP)-dependent protein kinase, which is involved in transcriptional control (reviewed in Nigg, 1990), is an example of a protein which is anchored to cytoplasmic membranes. cAMP-dependent protein kinases are composed of two regulatory subunits and two catalytic subunits, which together constitute an inactive holoenzyme. Binding of cAMP to the regulatory subunit dimer results in the release and activation of the catalytic subunits. The subcellular distribution of the inactive holoenzyme has been shown by subcellular fractionation and

immunofluorescent microscopy to be associated with the Golgi complex in epithelial cells and fibroblasts (Nigg *et al.*, 1985a). Treatment of cells with forskolin to increase the cAMP levels resulted in the dissociation of the catalytic subunit, which then predominantly accumulated in the nucleus. The regulatory subunit remained associated with the Golgi complex. The effect of forskolin was reversible in that after removal, the catalytic subunits reassociated with the Golgi complex in the cytoplasm (Nigg *et al.*, 1985b).

ii. RNA association with intracellular membranes

It is well known that a proportion of ribosomes in cells are associated with the endoplasmic reticulum (known as the rough endoplasmic reticulum). These ribosomes are involved in synthesising proteins destined for extracellular secretion, or for incorporation into the plasma membrane or intracellular organelles that comprise the endomembrane system. Ribosomes in the process of translating these proteins are targeted to the rough endoplasmic reticulum via recognition of a signal sequence contained within the nascent polypeptide. This sequence is recognised by a signal recognition particle, a complex that consists of one 7S RNA molecule and six different polypeptides (Walter *et al.*, 1981; reviewed in Walter and Johnson, 1994). The signal recognition particle then binds to a receptor on the endoplasmic reticulum, and the nascent polypeptide is translocated across the endoplasmic reticulum membrane (Walter and Blobel, 1981a,b). Thus, unlike polyribosome attachment to the cytoskeleton, association of ribosomes to the endoplasmic reticulum is not thought to be mediated by mRNA.

However, a role for mRNA binding to intracellular membranes has not been entirely ruled out. For example, early studies showed that in both human diploid fibroblasts and rat hepatocytes, significant amounts of mRNA remained attached to membranes after complete removal of ribosomes and their nascent polypeptides chains by treatment with high ionic strength buffer and puromycin (Lande *et al.*, 1975; Cardelli *et al.*, 1976). Treatment of these membranes with RNase and EDTA indicated that the poly(A), and possibly non-poly(A) portions of the mRNA were involved in binding to membranes (Milcarek and Penman, 1974; Lande *et al.*, 1975; Cardelli *et al.*, 1976). Also, dormant cysts of the brine shrimp *Artemia*, contain

latent, poly(A)-rich mRNA, which is to a large extent associated with cytoplasmic membranes (Grosfeld *et al.*, 1977; Simons *et al.*, 1978; Nilsson and Hultin, 1982). In addition, studies on protein targeting to the endoplasmic reticulum in *Saccharomyces cerevisiae* have shown that cells lacking the signal recognition particle and its receptor are viable, indicating that alternative pathways must exist (reviewed in Walter and Johnson, 1994). Although it is not known whether this alternative mechanism occurs co-translationally or post-translationally, it is possible that alternative pathways may be mediated by mRNA interactions.

Messenger RNA has also been hypothesised to be associated with the annulate lamellae. *In situ* hybridisation to poly(A) RNA in oocytes of the amphibian *Necturus* indicated that poly(A) was localised over cytoplasmic regions of annulate lamellae, suggesting maternal mRNA is associated with annulate lamellae (reviewed in Kessel, 1992). In addition, there have been numerous reports of a close relationship between the pores of annulate lamellae with polyribosomes and with fibrogranular bodies, which are possibly made up of mRNA, rRNA, tRNA and protein (Kessel, 1992). Since annulate lamellae are highly prevalent in amphibian oocytes, it has been postulated that the low efficiency of translation of maternal mRNAs is due to sequestering of the mRNA, and that annulate lamellae have a role in this sequestration (Shiokawa, 1983; Kessel, 1992).

III. Description of the Cytoskeleton and Membrane Systems in *Xenopus* Oocytes

Since immunocytochemistry has shown 7S RNPs to be distributed apparently homogeneously throughout the cytoplasm of oocytes (Mattaj *et al.*, 1983; Viel *et al.*, 1990), any factors involved in the retention of 7S RNPs would need to be distributed throughout the cytoplasm in a similar fashion. Thus, before investigating whether 7S RNPs are sequestered in the cytoplasm of previtellogenic oocytes due to an association with the cytoskeletal or internal membrane systems, it was first necessary to assess what components of these systems are present in oocytes. The following is a description of each of these systems throughout oogenesis, with an emphasis on previtellogenic stages.

i. The cytoskeleton

The presence of microtubules throughout oogenesis has been well documented using anti-tubulin immunofluorescence microscopy and laser scanning microscopy (Palacek *et al.*, 1985; Gard, 1991). Stage I oocytes contain a poorly ordered array of cytoplasmic microtubules, which is more dense in the cortical cytoplasm, surrounding the nucleus, and surrounding the mitochondrial mass (Palacek *et al.*, 1985; Gard, 1991). A radial tubulin array, emanating from the nucleus and extending throughout the cytoplasm to the periphery of the oocyte is first visible in stage II oocytes (Palacek *et al.*, 1985; Yisraeli *et al.*, 1990; Gard, 1991). As the oocyte develops and the nucleus is displaced towards the animal pole, the microtubule array becomes polarised: the radial array is still present, but only detectable in the animal hemisphere of stage VI oocytes. Microtubules are also present in the vegetal hemisphere, but appear less ordered, with a network of microtubules and microtubule bundles interspersed with the large yolk platelets (Palacek *et al.*, 1985; Yisraeli *et al.*, 1990; Gard, 1991). It has been estimated that there is about 0.12 μg (three percent of total soluble protein) tubulin per stage VI oocyte, and 25% of this is in polymeric form (Jesus *et al.*, 1987).

The presence of intermediate filaments is also well documented in oocytes. Examination of previtellogenic oocytes using electron microscopy has shown the presence of intermediate filaments crossing the cytoplasm in various directions (Godsave *et al.*, 1984a). These intermediate filaments were shown to be constituted of vimentin and cytokeratins. Early stage I oocytes first show a fine perinuclear ring of vimentin, and as the oocytes grow, more vimentin strands are found throughout the oocyte cytoplasm, forming a fine filamentous network. The mitochondrial mass and the perinuclear sphere of cytoplasmic masses also contain vimentin (Godsave *et al.*, 1984a). By late vitellogenesis, vimentin staining was shown to be asymmetric between the animal and vegetal poles. In the animal pole, vimentin is confined to the yolk-free areas of the cytoplasm that divide the yolk platelets into columns. In the vegetal hemisphere, vimentin is distributed in apparently random loci in the vegetal yolk mass and irregularly around the cortical cytoplasm (Godsave *et al.*, 1984a).

In stage I oocytes, cytokeratins are first found as sparse cortical threads and surrounding the mitochondrial mass (Godsave *et al.*, 1984b). During early

vitellogenesis, cytokeratins appear around the nuclear membrane and radially arranged filaments project towards the cortex, which become much more dense throughout vitellogenesis (Godsave *et al.*, 1984b). In mature oocytes, there is a distinct animal/vegetal polarity in cytokeratin organisation. In the vegetal region, filaments form a fine network characterised by distinct vertices and fine interconnecting fibrils (Klymkowsky *et al.*, 1987), and in the animal hemisphere fine filaments project radially from the nucleus through yolk-free tracks towards the cortex (Godsave *et al.*, 1984b).

The presence of actin filaments in *Xenopus* oocytes is less well characterised. In stage I oocytes actin cables were shown to course throughout the cytoplasm (reviewed in Gard, 1995). Stage VI oocytes have an extensive network of actin cables, which surrounds the germinal vesicle, and is present in yolk-free radii extending between the germinal vesicle and cortex, and also forms a dense three-dimensional network in the vegetal cytoplasm (Gall *et al.*, 1983; reviewed in Gard, 1995). Stage VI oocytes have been shown to contain 1.4-1.7 μg of actin per oocyte, although 75% of this does not sediment under forces that would pellet filamentous actin (Merriam and Clark, 1978). In addition, an actin shell was demonstrated around yolk platelets in unfertilised eggs (Colombo *et al.*, 1981). Thus, the three major cytoskeletal components are present in *Xenopus* oocytes, and are distributed throughout the cytoplasm of previtellogenic oocytes such that they could be involved in retention of 7S RNPs in the cytoplasm.

ii. The internal membrane system

Previtellogenic oocytes contain large amounts of endoplasmic reticulum, but only small, inconspicuous Golgi complexes. Scattered throughout the cytoplasm are lipids, small groups of mitochondria, and spherical structures consisting of annulate lamellae (Dumont, 1972). As well as mitochondria, the mitochondrial mass also contains endoplasmic reticulum and a few Golgi complexes. In later stage oocytes, membrane structures are restricted to the corridors of yolk-free cytoplasm which radiate outwards from the germinal vesicle, and to cortical regions of the oocyte (Bement and Capco, 1990). Thus it is also possible that 7S RNPs are sequestered by

interactions with these membrane components, as they appear to be present throughout the cytoplasm of previtellogenic oocytes.

5.2 RESULTS

Immunocytochemical studies have shown that 7S RNPs are found exclusively within the cytoplasm of oocytes (Mattaj *et al.*, 1983; Viel *et al.*, 1990). Although the distribution of 7S RNPs appeared homogeneous throughout the cytoplasm, these studies were not of high enough resolution to determine whether the 7S RNPs were associated with specific cytoplasmic structures. Thus, a series of biochemical fractionation techniques were used to investigate the association of 7S RNPs with cytoplasmic structures.

I. 7S RNPs are not Associated with the Oocyte Cytoskeleton

To determine whether endogenous 7S RNPs in the cytoplasm of previtellogenic oocytes are associated with cytoskeletal components, Triton X-100 extraction was performed on oocytes of different stages, first using the methods of Yisraeli *et al.* (1990). Whole oocytes were homogenised in Triton X-100 extraction buffer, and centrifuged to yield a pellet of insoluble, cytoskeletal factors and a soluble cytosolic supernatant fraction. The RNA was then isolated from each fraction, and the distribution of 5S RNA established by northern analysis using an antisense 5S RNA probe. Alternatively, the detergent insoluble and soluble fractions were subject to immunoprecipitation using an anti-TFIIIA antibody. The RNA was then extracted from the immunoprecipitate fractions and also analysed by northern analysis.

The results of a typical experiment are shown in Figure 5-1. In both previtellogenic and vitellogenic oocytes, the majority of total RNA is located in the detergent soluble fraction after Triton X-100 extraction (Fig. 5-1A). In previtellogenic oocytes (stages I and II), all of the endogenous 5S RNA was found in the detergent soluble fraction (Fig. 5-1B, cf. lanes 4 and 5). In late vitellogenic oocytes (stage V), when most of the stored 5S RNA has been released from binding TFIIIA and incorporated into ribosomal subunits, 5S RNA was again detected in the detergent

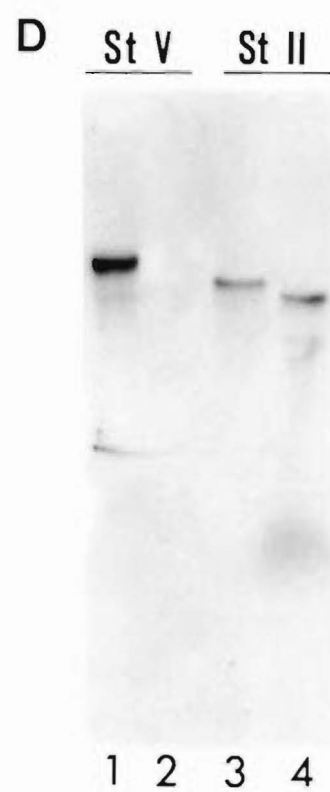
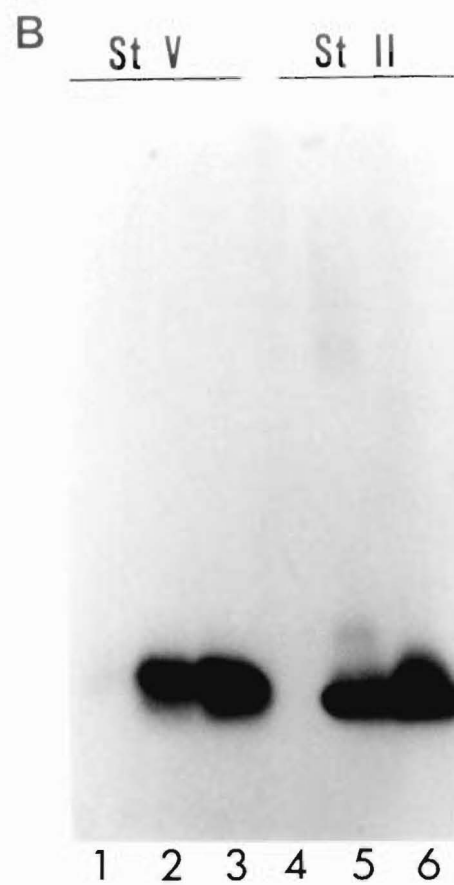
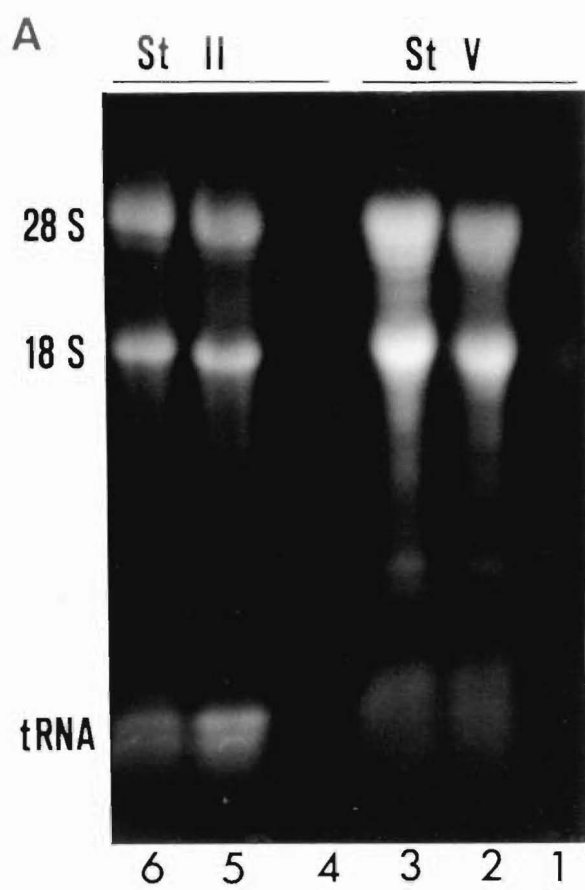
soluble fraction (Fig. 5-1B, cf. lanes 1 and 2). However, in some experiments over-exposures of films revealed a small amount of 5S RNA in the cytoskeletal fraction of stage V oocytes (data not shown). The antisense 5S RNA probe binds only to 5S RNA; no binding to any higher molecular weight RNA or to tRNA was ever observed (cf. Fig. 5-1A and B). As an additional control, an identical experiment to that shown in Figure 5-1B was performed using a sense-strand 5S RNA probe. There was no hybridisation to 5S RNA, showing that the antisense 5S RNA probe binds in a sequence-specific manner to 5S RNA (Fig. 5-1C).

As a control for the fractionation technique, identical aliquots of RNA isolated from cytoskeletal and soluble fractions were probed with the vegetally localised Vg1 antisense mRNA. As expected, all Vg1 mRNA in mature oocytes was present in the cytoskeletal fraction (Fig. 5-1D, cf. lanes 1 and 2). In previtellogenic oocytes, Vg1 mRNA was distributed in approximately equal amounts between the cytoskeletal and soluble fractions (lanes 3 and 4), in the same pattern observed by Yisraeli *et al.* (1990). The reason for the altered mobilities of the Vg1 transcript between lanes 2, 3 and 4 is unknown. The presence of Vg1 mRNA in the cytoskeletal fraction of previtellogenic oocytes also serves as an internal control, showing that the lack of 5S RNA in this fraction was not due to loss of the sample.

To provide further evidence that 7S RNPs were not present in the cytoskeletal fraction, and to determine if the faint amount of 5S RNA observed in the cytoskeletal fraction of stage V oocytes was in the form of 7S RNPs, immunoprecipitation assays were performed on detergent soluble and insoluble fractions after Triton X-100 extraction, using an anti-TFIIIA antibody. Figure 5-2 shows that for both previtellogenic and vitellogenic oocytes, 7S RNPs were only immunoprecipitated from the detergent soluble fraction (lanes 2 and 5). The slightly higher running band in lane 3 is probably due to impurities in the sample. The above experiments were repeated using oocytes from many different female frogs, and at no time were 7S RNPs ever detected in the cytoskeletal fraction.

The above results indicated that 7S RNPs were not present in the cytoskeletal fraction under the conditions of extraction used by Yisraeli *et al.* (1990). This procedure was optimised by these workers for the specific partitioning of Vg1 mRNA into the detergent insoluble fraction, and fibronectin mRNA, as a control, into the detergent soluble fraction. The extraction buffer contained 300 mM KCl, to

Figure 5-1 5S RNA is not contained within the cytoskeletal fraction of oocytes. Previtellogenic (St II) or vitellogenic (St V) oocytes were fractionated using Triton X-100 into cytoskeletal and soluble fractions. Total RNA was extracted from each fraction and separated by agarose gel electrophoresis and visualised by ethidium bromide staining and UV illumination (A), or transferred to nylon membrane and probed with antisense 5S RNA (B). Lanes 1 and 4, cytoskeletal fractions; lanes 2 and 5, detergent soluble fractions; lanes 3 and 6, total oocytes. A similar membrane was probed with sense-strand 5S RNA (C) or antisense Vg1 transcript (D). Lanes 1 and 3, cytoskeletal fractions; lanes 2 and 4, detergent soluble fractions.



solubilise yolk proteins (Yisraeli *et al.*, 1990). However, no attempt was made by these workers to determine what (if any) cytoskeletal components were present in the detergent insoluble fraction using this extraction procedure. To determine whether 7S RNPs are associated with cytoskeletal components not recovered using the above extraction procedure, I used a series of extraction protocols to specifically enrich each of the three main cytoskeletal components.

To specifically enrich oocyte microtubules, Triton X-100 extractions were carried out in the presence of 300 mM sucrose, which is known to stabilise microtubules (Suprenant, 1993). The distribution of 5S RNA after extraction with this protocol was similar to that described above, with 5S RNA present in the detergent soluble fraction only (data not shown). In addition, no differences were observed when extractions were carried out at 4°C versus room temperature (data not shown).

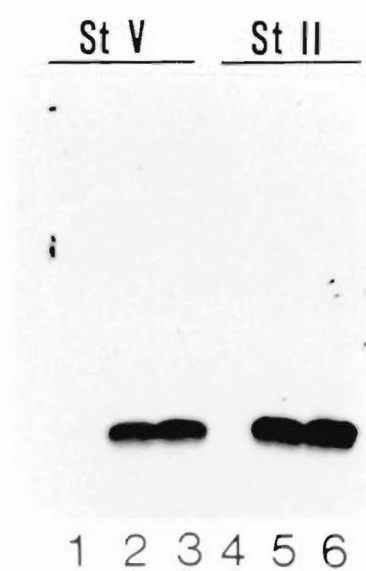
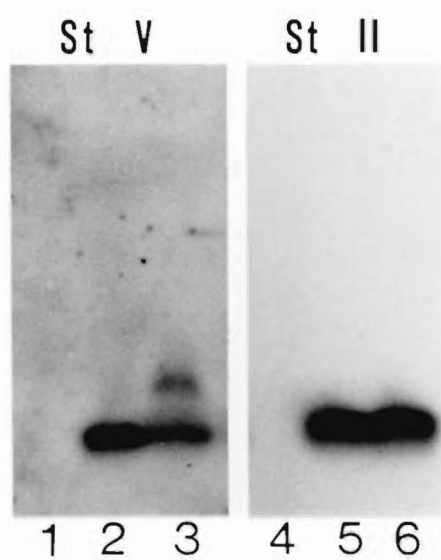
Microfilaments are known to be solubilised in buffers containing 130 mM KCl or greater (Vedeler *et al.*, 1991). To ensure the stabilisation of actin filaments, a low salt extraction procedure was performed. This extraction buffer was designed to resemble the intracellular ionic composition of *Xenopus* oocytes, and contained 92.5 mM KCl (Capco and Bement, 1991). 5S RNA distribution after total RNA extraction from fractions isolated using this protocol is shown in Figure 5-3. Both vitellogenic and previtellogenic oocytes show distinct 5S RNA bands in the cytoskeletal fraction (lanes 1 and 3), although the majority of 5S RNA is still in the detergent soluble fraction (lanes 2 and 4). Under these extraction conditions, the localisation of Vg1 mRNA was identical to that described above (data not shown).

To determine if the 5S RNA present in the cytoskeletal fraction after this low salt extraction was in the form of 7S RNPs, immunoprecipitation analyses were performed on each fraction, as shown in Figure 5-4. After extraction in low salt buffer, all 5S RNA immunoprecipitated with the anti-TFIIIA antiserum was in the detergent soluble fraction (lanes 1 and 2 and 4 and 5). In the above immunoprecipitation experiments, total oocyte fractions were homogenised in standard immunoprecipitation buffer (Ipp150), to show that homogenisation of oocytes in the various Triton X-100 extraction buffers did not impair the ability of 7S RNPs to be immunoprecipitated. This control clearly shows that there is no impairment in the immunoprecipitation of 7S RNPs after extraction in Triton X-100 buffer, since in all cases there are no significant differences between total oocyte

Figure 5-2 7S RNPs are not contained within the cytoskeletal fraction of oocytes. Vitellogenic (St V) or previtellogenic (St II) oocytes were fractionated into cytoskeletal and detergent soluble fractions and the fractions subject to immunoprecipitation with an anti-TFIIIA antibody. The RNA was extracted from immunoprecipitates, separated by agarose gel electrophoresis, transferred to nylon membrane and probed with antisense 5S RNA. Lanes 1 and 4, cytoskeletal fractions; lanes 2 and 5, detergent soluble fractions; lanes 3 and 6, total oocytes.

Figure 5-3 Small amounts of 5S RNA are contained in the cytoskeletal fraction after extraction with a low salt extraction buffer. Vitellogenic (St V) or previtellogenic (St II) oocytes were fractionated with a low salt Triton X-100 extraction buffer into cytoskeletal and detergent fractions, and the distribution of 5S RNA analysed as described in Figure 5-1. Lanes 1 and 3, cytoskeletal fractions; lanes 2 and 4, detergent soluble fractions.

Figure 5-4 7S RNPs are not contained within the cytoskeletal fraction after extraction with a low salt buffer. Vitellogenic (St V) or previtellogenic (St II) oocytes were fractionated into cytoskeletal and detergent soluble fractions, and the fractions subject to immunoprecipitation with anti-TFIIIA antisera. RNA was extracted from immunoprecipitates and analysed as described for Figure 5-2. Lanes 1 and 4, cytoskeletal fractions; lanes 2 and 5, detergent soluble fractions; lanes 3 and 6, total oocytes.



fractions and detergent soluble fractions (Fig. 5-2 and Fig. 5-4, cf. lanes 2 with 3 and 5 with 6).

One further extraction procedure was utilised, in which intermediate filaments were enriched in the detergent insoluble fraction. At high salt concentrations (1.5 M KCl) most oocyte proteins are solubilised, leaving an intermediate filament-enriched fraction (Pondel and King, 1988). Under these extraction conditions, as expected, all 5S RNA was found in the detergent soluble fraction (data not shown).

II. 7S RNPs are not Associated with Oocyte Intracellular Membranes

The above results indicated that 7S RNPs are not associated with any of the three main components of the cytoskeletal system in oocytes. To determine if 7S RNPs are associated with the internal membrane system in oocytes, an intracellular membrane fraction was prepared from oocytes using sucrose gradient centrifugation (Colman, 1984). Total RNA was then extracted from membrane and cytosol fractions, or fractions were subject to immunoprecipitation with anti-TFIIIA antiserum, followed by extraction of RNA and northern analysis to determine the distribution of 5S RNA.

Figure 5-5 shows the distribution of 5S RNA after total RNA extraction from membrane and cytosol fractions of previtellogenic (stages I and II) and vitellogenic (stage V) oocytes. These results show that a small amount of 5S RNA was distributed within the membrane fraction of both previtellogenic (lane 4) and late stage oocytes (lane 1), although most of the 5S RNA was in the cytosolic fractions (lanes 2 and 5). To determine the nature of the 5S RNA present in the membrane fractions, immunoprecipitation assays were performed, as shown in Figure 5-6. 5S RNA immunoprecipitated with the anti-TFIIIA antiserum was mostly present in the soluble cytosolic fraction of oocytes (lanes 2 and 5). These data show that the majority of 7S RNPs are not associated with the internal membrane system in oocytes, indicating that the mechanism of cytoplasmic retention is not primarily due to binding to insoluble cytoplasmic structures.

Figure 5-5 Small amounts of 5S RNA are associated with oocyte intracellular membrane fractions. Vitellogenic (St V) or previtellogenic (St II) oocytes were separated into membrane and cytosol fractions using sucrose gradient centrifugation, and the total RNA extracted from each fraction and separated by agarose gel electrophoresis. After transfer to nylon membrane, the distribution of 5S RNA was determined by probing with an antisense 5S RNA sequence. Lanes 1 and 4, membrane fractions; lanes 2 and 5, cytosol fractions; lanes 3 and 6, whole oocytes.

Figure 5-6 7S RNPs are not associated with oocyte intracellular membranes fractions. Vitellogenic (St V) or previtellogenic (St II) oocytes were separated into membrane and cytosol fractions, and each fraction was subject to immunoprecipitation with anti-TFIIIA antisera. RNA was extracted from immunoprecipitates, and the distribution of 5S RNA determined as described in Figure 5-5. Lanes 1 and 3, membrane fractions; lanes 2 and 5 cytosol fractions; lanes 3 and 6, whole oocytes.

St V St II



1 2 3 4 5 6

St V St II



1 2 3 4 5 6

5.3 DISCUSSION

The above results show that retention of 7S RNPs in the cytoplasm of previtellogenic oocytes is not due to their binding to insoluble cytoplasmic structures. 7S RNPs were not present in Triton X-100 insoluble fractions, under all conditions of extraction employed, indicating that sequestration in the cytoplasm is not due to an association with the cytoskeleton. The 5S RNA which was present in cytoskeletal fractions in some treatments possibly reflects RNA present in cytoskeletal-associated ribosomes. Isolation of oocyte intracellular membranes also showed that the majority of 7S RNPs were not associated with these structures. The small amount of 5S RNA that was present in the membrane fraction is also probably in the form of ribosomes, functioning in the synthesis of membrane and secretory proteins.

These results agree with the results from previous studies which indirectly suggested that binding to cytoplasmic structures was not the mechanism of cytoplasmic retention of 7S RNPs (Mattaj *et al.*, 1983; Mattaj, 1986). In these studies, homogenates of previtellogenic oocytes were injected into the cytoplasm of fully grown oocytes, and using immunocytochemistry, 7S RNPs were shown to diffuse uniformly throughout the cytoplasm, but not enter the nucleus (Mattaj *et al.*, 1983). Since 7S RNPs were freely diffusible within the cytoplasm, this was seen as evidence by these workers for cytoplasmic structures not having a role in retention. However, it would be unlikely that there would be a high enough concentration of a putative 7S RNP receptor at the site of microinjection to bind all injected 7S RNPs, making some diffusion within the cytoplasm very likely. Also, these assays were performed using fully grown oocytes, where the mechanism of cytoplasmic retention may not be identical to that occurring in previtellogenic oocytes. Since, as detailed in section 5.1, the composition and subcellular location of cytoskeletal and membraneous structures changes markedly during oogenesis, these assays did not adequately discount the possibility of interactions with these components having a role in retention of 7S RNPs.

Having ruled out a role for insoluble cytoplasmic structures, the question remains as to what is the mechanism for the retention of 7S RNPs in the cytoplasm of previtellogenic oocytes? The following sections describe four alternatives for the

regulation of the subcellular distribution of 7S RNPs, based on current knowledge of these processes. These include a lack of nuclear retention sites for 7S RNPs, the formation of 7S RNP dimers, the role of an inhibitory binding subunit, and post-translational modifications. The four possible mechanisms are summarised in Figure 5-7.

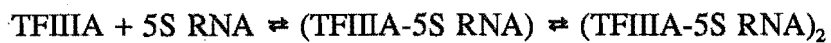
I. Lack of Nuclear Retention Sites for 7S RNPs

Recent studies have suggested that, unlike protein import, protein export from the nucleus does not require a nuclear export signal, but is instead the default pathway (Schmidt-Zachmann *et al.*, 1993). Nuclear accumulation of proteins is thought to result from intranuclear interaction with other nuclear factors (Schmidt-Zachmann *et al.*, 1993; Vancurova *et al.*, 1993; Paine *et al.*, 1995). Nuclear accumulation of RNA also appears to occur via interactions with other nuclear factors. The nuclear retention of U6 small nuclear RNA (Vankan *et al.*, 1990), U3 small nucleolar RNA (Terns and Dahlberg, 1994) and U8 small nucleolar RNA (Terns *et al.*, 1995) is saturable, indicating that localisation is due to the binding of some limiting nuclear factor (Boelens *et al.*, 1995; Terns *et al.*, 1995). The localisation of 7S RNPs in the cytoplasm could be explained by the hypothesis that once newly transcribed 5S RNA binds to TFIIIA in the nucleus, TFIIIA can no longer bind to the 5S RNA gene and, since it no longer has an intranuclear binding site, is exported to the cytoplasm. This idea is partly supported by the observation that excess 5S RNA inhibits transcription from 5S RNA genes (Pelham and Brown, 1980; Pelham *et al.*, 1981). In the above scenario, 7S RNPs would actually be capable of nuclear import, agreeing with the results of L. A. Allison (unpubl. observ.), where import of 7S RNPs was observed into somatic cell nuclei cultured *in vitro*. However, the 7S RNPs would be rapidly re-exported to the cytoplasm, as illustrated in Figure 5-7A, resulting in the predominant cytoplasmic location that is observed. This hypothesis could be tested by microinjecting 7S RNPs versus TFIIIA into the nucleus of oocytes, and analysing the subsequent cytoplasmic-nuclear distribution of the protein. If this hypothesis is correct, one would expect to see rapid

export of 7S RNPs to the cytoplasm, but retention of some TFIIIA in the nucleus due to binding to 5S RNA genes.

II. 7S RNP Dimerisation

Recent *in vitro* studies have investigated the assembly behaviour of 7S RNPs. Results indicated that complex formation is a reversible process, and at low concentrations (6 µg/ml), TFIIIA and 5S RNA may exist as free entities, but at higher concentrations (100 µg/ml) the one to one complex of TFIIIA and 5S RNA can self aggregate to form a dimer, illustrated by the following equation (Callaci *et al.*, 1990):



Since there is approximately 60 ng of TFIIIA per stage I to III oocyte (Shastry *et al.*, 1984), and the volume of stage I and II oocytes ranges from 0.5 to 47 nanolitres (calculated from diameters ranging from 100 to 450 µm; Dumont, 1972), this gives an intracellular concentration of TFIIIA ranging from 1 to 100 mg/ml, which is orders of magnitude larger than that required for dimerisation to occur. If dimerisation were to occur *in vivo*, this could explain cytoplasmic retention due to a masking of the TFIIIA NLS, as shown in Figure 5-7B. Dimer complexes would not be detected by traditional isolation methods, which would in effect, dilute the concentration of 7S RNP components, forcing the equilibrium of the above equation to the left, favouring the existence of monomer complexes as well as free protein and RNA components. The results of L. A. Allison (unpubl. observ.) could also be explained, since these assays used an overall concentration of 1.5 µg/ml protein, well below the concentration necessary for dimerisation. This hypothesis could perhaps be tested using cross linking studies to enable *in vivo* 7S RNP dimers to be detected.

III. Binding of an Inhibitory Subunit to 7S RNPs

There are many examples of cytoplasmic anchoring proteins playing a role in the regulation of nuclear localisation of transcription factors (reviewed in Hunt, 1989; Feldherr and Akin, 1994). One of the most extensively studied of these is NF- κ B, a transcription factor thought to be involved in the transcriptional activation of more than twenty genes (reviewed in Schmitz *et al.*, 1991). NF- κ B is a heterodimer composed of 50 (p50) and 65 (p65) kD subunits and, in most cell types, resides in an inactive form in the cytoplasm. DNA binding is induced by exposure of cells to a variety of agents, such as viruses (reviewed in Schmitz *et al.*, 1991). NF- κ B DNA binding activity is also detected after treatment of cells with deoxycholate, which has been shown to release an inhibitory factor, I κ B (Baeuerle and Baltimore, 1988a,b). Direct evidence for the role of I κ B in blocking nuclear import of NF- κ B came from studies where p65, p50 and I κ B were introduced into Vero cells. When over-expressed individually, all entered the nucleus; however, when p65 or p50 were over-expressed in the presence of I κ B, nuclear import was blocked (Zabel *et al.*, 1993). Furthermore, the presence of I κ B was shown to block binding of antibodies specific for the NLS region of p50 and p65, indicating that the inhibitor acts by masking the NLS (Zabel *et al.*, 1993).

An analogous situation to that of NF- κ B occurs in *Drosophila*, showing that this mechanism of regulation of nuclear import is common to a diverse range of eukaryotes. The dorsal protein is a transcription factor which functions in the formation of the dorso-ventral axis in the *Drosophila* embryo (reviewed in Courey and Huang, 1995). It can act as either an activator or repressor of a number of genes, depending on its concentration in the nucleus. A nucleocytoplasmic gradient exists in the blastoderm between the ventral pole, where dorsal is primarily nuclear, and the dorsal pole, where it is localised in the cytoplasm (reviewed in Hunt, 1989; Schmitz *et al.*, 1991). Although the establishment of this gradient is a complex process, involving several different genes, the protein cactus, which shows sequence similarity to I κ B (Geisler *et al.*, 1992), seems to play an important role in regulating nuclear localisation. In mutants lacking cactus, dorsal is nuclear in both dorsal and ventral regions (Roth *et al.*, 1991). Further studies have suggested that the binding of cactus

to dorsal retains dorsal in the cytoplasm (Isoda *et al.*, 1994; reviewed in Courey and Huang, 1995).

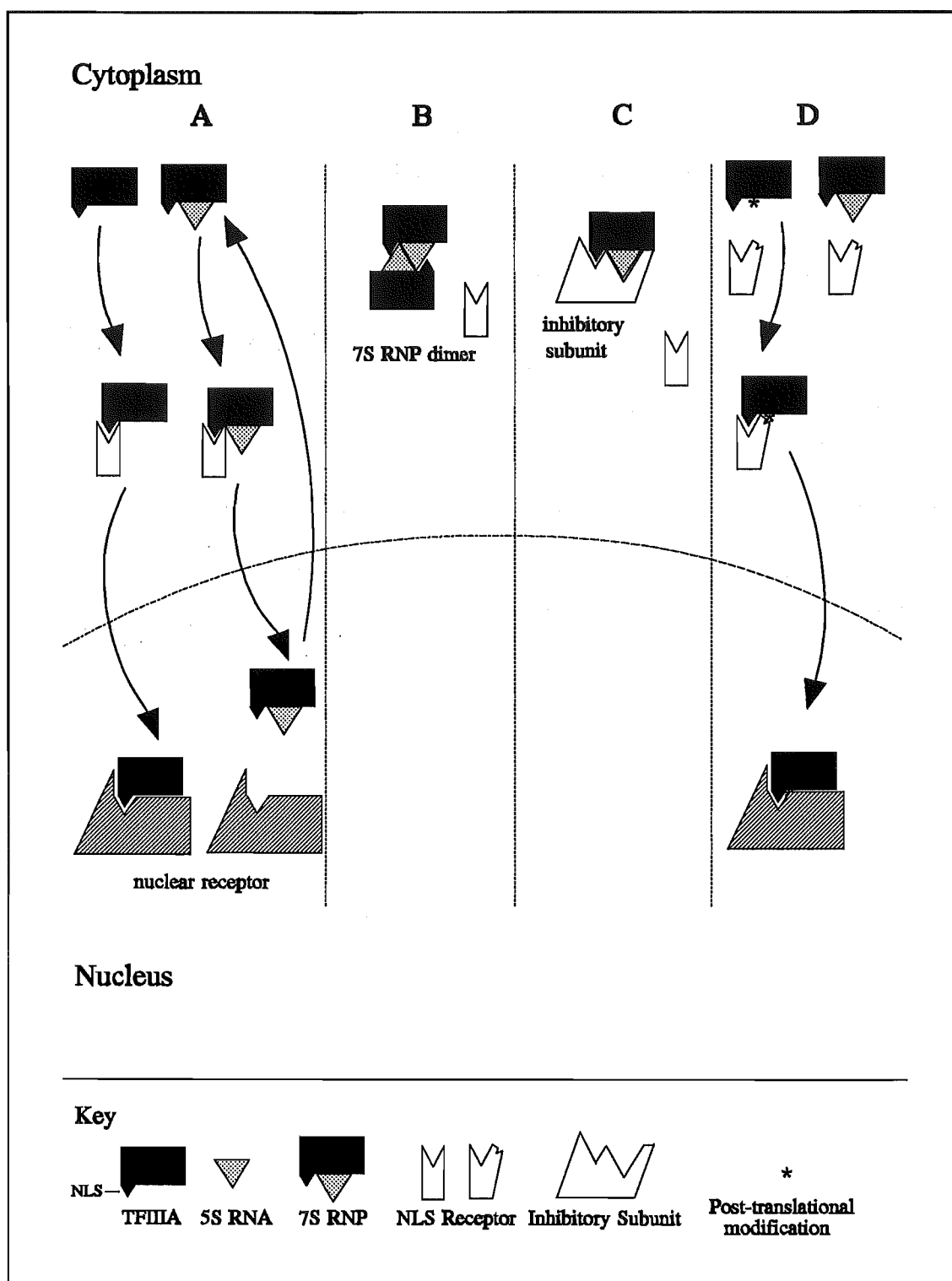
Thus, a possible mechanism for the cytoplasmic retention of 7S RNPs could be the binding of an inhibitory subunit protein which could prevent nuclear import, perhaps by masking the putative TFIIIA NLS (Fig. 5-7C). However, as 5S RNA bound to TFIIIA in oocytes has been found to consistently sediment at 7S, and not in higher molecular weight fractions, this mechanism seems unlikely. In addition, immunoprecipitations performed using anti-TFIIIA antibodies did not co-precipitate any other proteins (pers. observ.).

IV. Post-translational Modification

Yet another mechanism for the regulation of nuclear import of proteins is post-translational modification (reviewed in Feldherr and Akin, 1994). Studies of the nuclear import of hybrid proteins fused with the SV-40 T-antigen showed that the rate of nuclear import is significantly enhanced when residues flanking the NLS are included in the construct (Rihs and Peters, 1989). This increase in the rate of transport was shown to be due to phosphorylation at either of two positions by casein kinase II (Rihs *et al.*, 1991). Phosphorylation also appears to regulate the cell cycle-dependent nuclear transport of the yeast transcription factor SW15. When three serine residues adjacent to the NLS are phosphorylated, SW15 is cytoplasmic. Dephosphorylation prior to G1 is a prerequisite for nuclear import (reviewed in Feldherr and Akin, 1994). Thus, phosphorylation can both positively and negatively affect the regulation of nuclear import.

Thus, the mechanism for the localisation of TFIIIA in oocytes may be post-translational modification. For example, as shown in Figure 5-7D, TFIIIA may require this modification for high affinity interaction with nuclear import factors. The binding of 5S RNA might reduce or block the modification, resulting in a molecule with less or no affinity for import factors, that therefore remains cytoplasmic.

Figure 5-7 Possible mechanisms for the cytoplasmic localisation of 7S RNPs. (A) 7S RNPs are capable of nuclear import, but do not accumulate as there is no nuclear retention site. On the left, the pathway taken by TFIIIA is shown, whereby nuclear import is mediated by a receptor molecule which recognises the putative TFIIIA NLS. TFIIIA is then retained in the nucleus via interactions with the 5S RNA gene, or other nuclear receptors. 7S RNPs are imported into the nucleus, also via interactions with the NLS receptor, but since they cannot interact with the nuclear receptor(s), are rapidly exported to the cytoplasm. (B) Nuclear import of 7S RNPs is prevented by the formation of 7S RNP dimers. The formation of dimers is dependent on sequences contained within both protein and RNA moieties of 7S RNPs, and is concentration dependent. The TFIIIA NLS is thus masked, preventing interaction with the NLS receptor, so nuclear import does not occur. (C) Nuclear import of 7S RNPs is prevented by the binding of an inhibitory subunit. The NLS is masked from interaction with the NLS receptor, so nuclear import is prevented. (D) Post-translational modification (*) of TFIIIA is necessary for nuclear import. The post-translational modification is necessary for high affinity interaction with the NLS receptor. Binding of 5S RNA to TFIIIA reduces the ability of TFIIIA to be modified, reducing the affinity for the NLS receptor, and nuclear import.



However, there is no evidence that TFIIA is phosphorylated in oocytes (Kim *et al.*, 1990), although experiments have shown that it does not appear to be glycosylated (Jackson and Tijan, 1988). Two forms of TFIIA have been detected in somatic cells, one of which is two kD larger than the TFIIA found in oocytes, but this larger form is thought to arise from initiation of transcription 200 base pairs upstream from the start site of the smaller form, rather than from post-translational modifications (Kim *et al.*, 1990).

Further studies are clearly necessary to understand the mechanisms by which 7S RNP particles are restricted to the cytoplasm in previtellogenic oocytes. It is possible that there is not just one major mechanism responsible, and that different factors, such as those outlined above, may contribute to effect the cytoplasmic localisation that is observed.

V. Cytoplasmic Retention of other Molecules in *Xenopus*

Regulation of nuclear import is not only restricted to 7S RNPs during early *Xenopus* development. During oogenesis, the *Xenopus* nucleus acquires many proteins, several of which are stored for later use in embryogenesis. During oocyte maturation and germinal vesicle breakdown, these proteins are released into the cytoplasm. Because of the huge size of the oocyte nucleus, the sum of all the volumes of all nuclei during cleavage and the early blastula stages of embryogenesis is smaller than the original germinal vesicle volume, and it is not until mid-blastula that the total volume of all nuclei of the embryo add up to that of the original germinal vesicle. Thus, during early embryogenesis, the oocyte nuclear proteins are not all located in the nucleus. It has been observed that during this stage of development not all of these proteins behave the same with respect to nucleocytoplasmic localisation. Some proteins re-enter the embryonic nuclei immediately after fertilisation, while others are excluded from nuclei until blastula or neural stages of development (Dreyer *et al.*, 1986).

The mechanism of this regulation of nuclear import has been further investigated for the protein xnf7 (*Xenopus* nuclear factor 7), which re-enters embryonic nuclei at the mid-blastula stage. The release of xnf7 from the germinal

vesicle at oocyte maturation coincides with phosphorylation, while re-accumulation into blastula nuclei correlates with an increase in abundance in the less phosphorylated isoforms of the protein (Miller *et al.*, 1991). In addition, a 22 amino acid sequence was recently identified which functions cooperatively with two phosphorylation sites within the xnf7 molecule to retain the molecule in the cytoplasm. Addition of a second NLS to the protein did not relieve the cytoplasmic localisation, suggesting retention is not due to a masking of the NLS, but involves an anchoring mechanism where the 22 amino acid sequence interacts with an anchor protein (Li *et al.*, 1994). Interestingly, xnf7 possesses several zinc-finger-like regions, and two acidic regions similar to transcriptional activating domains. In addition, it is thought to bind to double stranded DNA, suggesting it might function as a transcription factor (Reddy *et al.*, 1991).

An analogous situation to TFIIIA and 5S RNA in oocytes occurs with mRNA. During oogenesis, over 80% of the mRNA synthesised is sequestered into messenger ribonucleoprotein particles (mRNPs) and stored in the cytoplasm in a translationally repressed state. The two major proteins in these mRNPs are mRNP3 and mRNP4 (Darnbrough and Ford, 1981). Unexpectedly, sequencing of mRNP4 showed that it is identical to FRG Y2, a germ cell-specific factor stimulating transcription from Y-box promoters (Murray *et al.*, 1992; Deschamps *et al.*, 1992). Thus, analogous to TFIIIA, mRNP4/FRG Y2 has the dual function in oocytes of promoting transcription from a specific set of genes, and associating with a broad spectrum of mRNAs so they are masked from the translation machinery (Ranjan *et al.*, 1993; Tafuri and Wolffe, 1993; Bouvet and Wolffe, 1994). As with TFIIIA, mRNP4/FRG Y2 performs its role of transcription in the nucleus, but is stored bound to mRNA in the cytoplasm. The precise determinants and mechanisms of control of the compartmentalisation of this molecule also have not yet been defined.

The above data indicate that 7S RNPs are not the only molecules which are restricted to the cytoplasm in a regulated manner during early development in *Xenopus*. Thus, further investigation of the mechanism by which 7S RNPs are sequestered in the cytoplasm may provide insights into the mechanisms of localisation of these other molecules, and to the regulation of nuclear import and transcription in general.

CHAPTER 6

GENERAL DISCUSSION

After synthesis in previtellogenic *Xenopus* oocytes, 5S RNA is exported to the cytoplasm where it is stored in one of two ribonucleoprotein particles. At the onset of vitellogenesis, 5S RNA moves back into the nucleus where it is eventually targeted to the nucleolus for incorporation into 60S ribosomal subunits. The results presented in this study provide further information on some of the steps taken in this pathway.

6.1 REQUIREMENTS FOR THE PATHWAY TAKEN BY 5S RNA DURING OOGENESIS

Out of 32 mutant 5S RNA molecules tested, only one, mutant 10-13 was defective for TFIIA binding *in vivo*. This mutation contains base substitutions in loop A, a region thought to be important for maintaining the tertiary structure of 5S RNA (Romaniuk, 1989; Baudin *et al.*, 1991), and which was also shown to have a low *in vitro* binding affinity for TFIIA (Romaniuk, 1989). Interestingly, other 5S RNA mutants which showed *in vitro* binding affinities for TFIIA similar to or lower than that of mutant 10-13 were capable of binding TFIIA *in vivo*. This result reflects the differences between *in vitro* assays and the situation *in vivo*, where TFIIA is not the only 5S RNA-binding protein, and where the exogenous mutant

5S RNAs must also compete with endogenous 5S RNA. A comparison of the measured competition strengths of the various 5S RNA mutants for TFIIA binding reveals that mutant 10-13 shows a significantly lower competition strength than any of the other mutants (Baudin and Romaniuk, 1989; Romaniuk, 1989; Baudin *et al.*, 1991). Thus, the measurement of competition strength appears to give a more accurate indication of RNP formation *in vivo*, and helps to explain why 10-13 was the only mutant not detectable in 7S RNPs *in vivo*.

None of the mutants tested, including mutant 10-13, were defective in binding to ribosomal protein L5. These results are consistent with the *in vitro* binding data for these mutants, where the majority of mutants bound to L5 with near wild type affinity (Q. You, W. Q. Zang, and P. J. Romaniuk, in prep.). Deletion of the bulged nucleotide at position 63 also did not affect L5 binding, although the equivalent nucleotide is necessary for binding of the *E. coli* 5S RNA-binding protein, L18 to 5S RNA (Peattie *et al.*, 1981; Christiansen *et al.*, 1985).

The above results suggest that different structural features of the 5S RNA molecule are important for binding TFIIA and L5. This is not surprising since the two proteins share no sequence homology and are thought to bind to 5S RNA via different mechanisms. TFIIA contains an array of nine tandem repeats of a zinc binding domain characterised by invariant cysteines and histidines, termed zinc finger motifs (Miller *et al.*, 1985). Although the mechanism has not yet been deduced, these zinc finger motifs are responsible for the specific DNA and RNA binding activities of this protein (Theunissen *et al.*, 1992; Clemens *et al.*, 1993; reviewed in Pieler and Theunissen, 1993). In contrast, ribosomal protein L5 does not contain zinc finger motifs, but does contain a region of basic amino acids at the carboxy-terminus that is conserved from yeast to mammals (Yaguchi *et al.*, 1984; Chan *et al.*, 1987; Kenmochi *et al.*, 1991). These basic amino acids were shown to have a key role in the binding of the yeast 5S RNA-binding protein, L1 to 5S RNA (Yaguchi *et al.*, 1984; Yeh and Lee, 1995b).

Four of the 5S RNA mutants tested were not detected in 60S ribosomal subunits. These were mutant 10-13, mutant 96-101, in which non-canonical base pairs were replaced with Watson-Crick forming base pairs, and mutants $\Delta 49,50$ and $\Delta 63$, in which bulged nucleotides in helices III and II were deleted, respectively. These results indicate that binding to L5 is not sufficient for assembly of 5S RNA

into 60S subunits, since all of the mutants defective for ribosome assembly were shown to form 5S RNPs. As discussed in Chapter 3, defective ribosome assembly could be due to a loss of recognition features necessary for interactions with other ribosomal components, or due to an inherent instability of 60S subunits containing these RNAs because of conformational rearrangements or an inability to associate with 40S subunits. Support for the former hypothesis comes from studies in yeast, where it appeared that mutant 5S RNAs were stable once incorporated into 60S ribosomal subunits, and no degradation of assembled subunits was observed (Van Ryk *et al.*, 1992). Alternatively, the lack of ribosome incorporation exhibited by these mutants may have been due to a defect in an earlier step of the 5S RNA pathway, such as nuclear import or nucleolar targeting. However, all of the mutants were previously shown to be capable of nuclear import (Allison *et al.*, 1993; L. A. Allison and P. J. Romaniuk, in prep.), ruling out this step as a reason for the lack of ribosome incorporation. Likewise, the results presented in Chapter 4 demonstrated that a defect in nucleolar localisation was not the reason for the inability of these mutants to be incorporated into ribosomes. Mutants 10-13 and 96-101 were both localised to nucleoli to some degree, indicating defective ribosome incorporation is due to some later step in the assembly process. Mutants $\Delta 49,50$ and $\Delta 63$ showed some localisation over nucleoli in the *in situ* localisation assays, however, this was nowhere near the extent indicated by the biochemical fractionation assay. These results implied that $\Delta 49,50$ and $\Delta 63$ are associated with high molecular weight structures in the nucleus so that they are pelleted in the biochemical fractionation of nucleoli. It is possible therefore, that $\Delta 49,50$ and $\Delta 63$ are prevented from ribosome incorporation because of formation of these large complexes.

Together with previous analyses on the nuclear import ability of these mutants, the results presented here also provide insights into cellular factors which are necessary for the different steps taken by 5S RNA in oocytes. Regions of the 5S RNA molecule found to be most important for nuclear import were helices II and V, and loops C and E (Allison *et al.*, 1993; L. A. Allison and P. J. Romaniuk, in prep.). These regions are different from those found to be required for TFIIIA binding and for ribosome assembly. It is therefore likely that different cellular factors are required for nuclear transport and for ribosome assembly of 5S RNA. Since mutant 10-13 was capable of nuclear import, this suggests that TFIIIA binding is not

a requirement for this step. It has previously been suggested that the binding of L5 to 5S RNA in the cytoplasm of oocytes mobilises 5S RNA for nuclear transport and nucleolar localisation (Allison *et al.*, 1991). Furthermore, preassembled 5S RNPs were shown to be imported into nuclei at a faster rate and to a greater extent than free 5S RNA, suggesting 5S RNP formation is a precursor step for nuclear import (K. J. Murdoch and L. A. Allison, submitted). Since all of the mutants were able to bind L5, the absolute requirement for this protein in nuclear import and for nucleolar targeting has yet to be demonstrated. The results presented here do suggest, however, that L5 binding is not sufficient for nucleolar localisation. In summary, a picture emerges whereby different structural features of 5S RNA, and different oocyte factors are required for the individual steps taken by 5S RNA during oogenesis.

6.2 SUBCELLULAR LOCALISATION OF 5S RNA

The results presented in Chapter 4 showed that only one third of endogenous 5S RNA and microinjected oocyte-type 5S RNA were associated with nucleoli in the *Xenopus* oocyte nucleus. Similarly, microinjected ribosomal protein L5 showed only slightly higher nucleolar localisation than oocyte-type 5S RNA. These results indicate that there is a pool of 5S RNA and L5 in the nucleus, and immunoprecipitation assays confirmed that a proportion of these were associated in the form of 5S RNPs. Since only a small amount of 5S RNA in the nucleus was immunoprecipitated with anti-TFIIIA, anti-L5 and anti-60S ribosomal subunit antisera, these results implied that 5S RNA is associated with other factors in the oocyte nucleoplasm. These may be part of nuclear import complexes, may function in retaining an excess of 5S RNA in the nucleus so it is readily available for ribosome assembly, or may be important in regulating the amount of 5S RNA at the nucleolus at one time.

The mechanism by which 7S RNPs are retained in the cytoplasm of previtellogenic oocytes remains to be elucidated. The results presented in Chapter 5 rule out the possibility that retention is due to association with cytoskeletal or internal membrane structures. Alternative mechanisms suggested include the role of putative inhibitory binding subunits, 7S RNP dimerisation, post-translational modifications, or simply a lack of nuclear retention sites for 7S RNPs.

Since mutant 10-13 was shown to be defective for binding TFIIIA *in vivo*, it would be interesting to determine the subcellular distribution of this mutant in previtellogenic oocytes. Furthermore, mutant TFIIIA molecules, which are defective in either RNA or DNA binding could provide information on the mechanism of cytoplasmic retention.

6.3 BEHAVIOUR OF SOMATIC-TYPE 5S RNA IN OOCYTES

Although somatic-type 5S RNA is synthesised in oocytes (Ford and Southern, 1973), it was previously not detected in long term storage particles or ribosomes (Denis and Wegnez, 1977). More recently, somatic-type 5S RNA was shown to be imported into the nucleus at a faster rate and to a greater extent than oocyte-type, and more was assembled into 60S ribosomal subunits after injection into the oocyte cytoplasm (Allison *et al.*, 1995). This was explained by the different protein binding activities of the two RNAs in the cytoplasm. Oocyte-type was predominantly associated with TFIIIA, whereas somatic-type 5S RNA preferentially associated with ribosomal protein L5. These results suggested that oocyte-type 5S RNA is more adapted for storage, whereas somatic-type is rapidly mobilised and assembled into ribosomes.

The nucleolar localisation results described in this study support this model. While only one third of oocyte-type was associated with nucleoli, over two thirds of somatic-type 5S RNA was nucleolar. Thus, even after import into the nucleus, which is the first step for incorporation into 60S ribosomal subunits, oocyte-type 5S RNA appears to be "stored," whereas somatic-type is localised over nucleoli, and therefore being assembled into ribosomal subunits. Interestingly, this result was not explained by differences in L5 binding between the two RNAs in the nucleus, since both were predominantly immunoprecipitated with anti-L5 antiserum. This indicates that other nuclear factors directly recognise the different sequences of oocyte-type and somatic-type 5S RNAs. These factors could be other nucleolar/ribosomal components which have a higher affinity for somatic-type 5S RNA, or nucleoplasmic factors which have a higher affinity for oocyte-type 5S RNA. These results are incorporated into a model

presented in Chapter 4 for the pathway taken by 5S RNA in the nucleus of *Xenopus* oocytes.

Xenopus somatic-type 5S RNA is more similar in sequence to mammalian 5S RNA than oocyte-type, and it has been suggested that the mutations which have occurred in oocyte-type are unlikely to be neutral (Ford and Southern, 1973). It appears that oocyte-type 5S RNA is specialised for the unusual route taken by this molecule during oogenesis, which includes bidirectional nuclear transport and long term storage in the cytoplasm, and perhaps storage in the nucleus.

6.4 THE PATHWAY TAKEN BY 5S RNA

The complicated pathway taken by 5S RNA in amphibian oocytes is largely thought to be peculiar to this cell type. In somatic cells, 5S RNA is generally thought to be targeted directly to nucleoli after synthesis, and assembled into 60S ribosomal subunits. However, a re-evaluation of the literature suggests 5S RNA may also follow a more complex route in somatic cells.

Studies in HeLa cells showed that up to 25% of 5S RNA is not associated with ribosomes, representing a pool of 5S RNA in the nucleus (Knight and Darnell, 1967). In addition, it has been shown that four times as much 5S RNA than 28S rRNA is synthesised in exponentially growing HeLa cells, far more than is required for ribosome assembly (Leibowitz *et al.*, 1973). Steitz *et al.* (1988) showed that 50% of the non-ribosome associated 5S RNA in HeLa cells was bound to L5, as a 5S RNP. Further studies showed that a fraction of 5S RNA in HeLa cells was precipitable with antibodies specific for a 37 kD HeLa protein, which is antigenically related to *Xenopus* TFIIIA (Lagaye *et al.*, 1988). 5S RNA bound to this protein was found in both nuclear and cytoplasmic fractions. These workers suggested that excess 5S RNA binds to the 37 kD protein, and is then targeted to the cytoplasm for degradation.

In both HeLa cells and rat liver cells, kinetic labelling studies detected 5S RNA in soluble, cytoplasmic fractions immediately after synthesis (Leibowitz *et al.*, 1973; Ogata *et al.*, 1993). In one of these studies, the cytoplasmic 5S RNA was proposed to re-enter the nucleus and be incorporated into 60S subunits

(Leibowitz *et al.*, 1973), but in the other study, the cytoplasmic 5S RNA was thought not to be a precursor to ribosomal 5S RNA, and to remain in the cytoplasm (Ogata *et al.*, 1993).

Taken together, the above data suggest that the pathway taken by 5S RNA in somatic cells may not be as straight forward as initially thought. Additional steps, such as a detour to the cytoplasm, similar to that occurring in *Xenopus* oocytes, may also be relevant to other cell types. The 5S RNA in the cytoplasm of somatic cells may simply be excess RNA targeted for degradation, or may have some other function, an idea which is partially suggested by its association with a range of cellular factors, described in the following section.

6.5 5S RNA/L5-CONTAINING COMPLEXES

5S RNA has been found in a number of RNP complexes in both oocytes and somatic cells, as summarised in Table 6-1. As well as 7S RNPs, 5S RNA is stored in 42S RNPs in previtellogenic oocytes. The 5S RNA-binding protein in 42S RNPs is p43, a protein which contains nine zinc finger domains that show homology to TFIIIA (Joho *et al.*, 1990). The other 42S RNP protein is p48, which binds to aminoacyl tRNA. p48 is structurally homologous to EF-1 α , an elongation factor which functions in transferring aminoacyl-tRNAs to the ribosome (Viel *et al.*, 1990; Coppard *et al.*, 1991). Evidence that aminoacyl-tRNA is transferred from 42S RNPs to ribosomes (le Maire and Denis, 1987) led to the suggestion that p48 is a stage-specific elongation factor. In addition, a small fraction of tRNA molecules purified from 42S RNPs were shown to carry a peptide or protein chain, suggesting that 42S RNPs carry out a ribosome-independent, incorporation of amino acids into protein (Denis and le Maire, 1987).

5S RNA, tRNA and mRNA have also been detected in RNPs sedimenting at 100-200S in previtellogenic *Xenopus* oocytes (Denis and le Maire, 1987). This fraction contained few, if any polysomes and was not simply aggregates of 42S RNPs. The function of these large particles remains to be established.

As mentioned in Chapter 4, certain 5S RNA variants have been found associated with the Ro autoantigen in *Xenopus* oocytes. These 5S RNA variants

Table 6-1 5S RNA and/or L5-containing complexes

Complex components	Species/cell type	Possible functions	References
5S RNP: 5S RNA, L5	HeLa cells <i>Xenopus</i> oocytes <i>Xenopus</i> embryos Yeast	Precursor to ribosome assembly Nuclear import Nucleolar targeting	Steitz et al., 1988 Allison et al., 1991; 1995 Wormington, 1989 Deshmukh et al., 1993 Allison et al., 1991; 1995 Steitz et al., 1988
7S RNP: 5S RNA, TFIIIA	<i>Xenopus</i> oocytes HeLa cells	Storage Degradation Nuclear export	Picard and Wegnez, 1979 Lagaye et al., 1988 Guddat et al., 1990
42S RNP: 5S RNA, p43, tRNA, p48	amphibian oocytes	Storage Delivery of aminoacyl-tRNAs to ribosome Peptide bond formation	Picard et al., 1980 le Maire and Denis, 1987 Denis and le Maire, 1987
100-200S RNP: 5S RNA, tRNA, mRNA	<i>Xenopus</i> oocytes	Unknown	Denis and le Maire, 1987
mutant 5S RNAs, Ro autoantigen	<i>Xenopus</i> ovary	5S RNA quality control pathway	O'Brien and Wolin, 1994
5S RNA, L5, 5.8S rRNA	rat liver	Unknown	Metspalu et al., 1980
5S RNA, L5, tRNA synthetases	rat liver	Aminoacylation of tRNA	Ogata et al., 1991a,b Ogata et al., 1994 Ogata et al., 1995
5S RNA, L5, mdm-2/mdm-2-p53	mouse fibroblast	Unknown	Marechal et al., 1994
L5, protein phosphatase type 1	chicken gizzard	Activate phosphatase activity	Hirano et al., 1995

contain one or more point mutations as well as additional 3' nucleotides (O'Brien and Wolin, 1994). Since these mutant 5S RNAs were inefficiently processed and eventually degraded, it was suggested that Ro may function as part of a quality control or discard pathway for 5S RNA production.

In preparations of rat liver cytosol, 5S RNPs were found associated with a macromolecular complex containing nine tRNA synthetases (Ogata et al., 1991a), and

with a smaller complex containing threonyl- and histidyl-tRNA synthetases (Ogata *et al.*, 1994). 5S RNA was shown to interact with methionyl-tRNA and methionyl-tRNA synthetase in the macromolecular aminoacyl synthetase complex (Ogata *et al.*, 1995). Furthermore, 5S RNPs enhanced the activities of some of the tRNA synthetases within the macromolecular complex (Ogata *et al.*, 1991b), and the threonyl-tRNA synthetase activity of the threonyl-histidyl-tRNA synthetase complex (Ogata *et al.*, 1994). 5S RNA in rat liver cytosol was also shown to form a ternary complex with L5 and 5.8S rRNA *in vitro* (Metspalu *et al.*, 1980).

The protein product of the oncogene mdm-2 is known to associate with p53, a protein involved in the response to DNA damage, giving rise to G1 arrest or cellular apoptosis. In mouse 3T3 cells which over-express mdm-2, 5S RNPs were found associated with mdm-2 and with the mdm-2-p53 complex (Marechal *et al.*, 1994). Finally, ribosomal protein L5 was shown to be associated with the catalytic subunit of the type 1 protein phosphatase, and also activated the phosphatase activity of a myosin-bound phosphatase, and the type 1 phosphatase catalytic subunit (Hirano *et al.*, 1995). It was suggested by these authors that L5 may target the phosphatase to the ribosome for dephosphorylation of ribosomal proteins, or to the mdm-2-p53 complex for dephosphorylation of p53.

The functional significance of most of these 5S RNA/L5-containing complexes is largely unknown and remains enigmatic. Possible functions could include as regulators or mediators of specific steps in ribosome biosynthesis or protein synthesis, or could be totally unrelated. Further research should clarify the roles of these complexes, and provide insights into the processes of ribosome biogenesis and protein synthesis in both oocytes and somatic cells.

ACKNOWLEDGEMENTS

I thank my supervisor of studies, Liz Allison, for her help and support over the time of this thesis. I am also grateful to my secondary supervisor, Drusilla Mason, for constructive comments on the final draft of this thesis. Thanks also to Frank Sin for providing encouragement and advice over the years.

I am deeply grateful for the expert technical guidance I received from Jan McKenzie in microscopy, photomicroscopy, thesis aesthetics and back care. Thanks also to Terry Williams for printing of photo plates. I am thankful to many staff members of the Department of Zoology who provided assistance in times of crisis, including Lyn de Groot, Franz Ditz, Nick Etheridge, Dave Greenwood, Bruce Lingard, Linda Morris, Tracey Robinson, John Scott and Roy Thompson.

I also thank my colleagues in our laboratory for their help and discussions, including Anne Bartley, Jenny Khoo, Ujjal Mukhopadhyay, Kirstie Murdoch, Sylvia Nagl, Jane Symonds and Seumas Walker.

Most importantly, thanks to my partner, Roger Brough for his constant support and patience, for help with figures and for providing numerous computing and word processing tips.

My PhD research was funded by a University of Canterbury Postgraduate Scholarship and a Ministry of Research, Science and Technology Postgraduate Study Award. I would like to acknowledge the Canterbury branch of the Royal Society, the Health Research Council of New Zealand, the NZ Federation of University Women and the New Zealand Society of Biochemistry and Molecular Biology for providing travel grants to enable me to attend two international conferences during my research.

Finally, I am grateful for the generous donations of plasmids and antisera from Susan Gerbi, Dave Goldfarb, Marc le Maire, Douglas Melton, Paul Romaniuk, Marion Schmidt-Zachmann and Mike Wormington which made many of the experiments in this thesis possible. Special thanks to Michael Ezrokhi for preparation of the U3 snoRNA template.

REFERENCES

- Adachi, Y., Copeland, T. D., Hatanaka, M. and Oroszlan, S.** 1993. Nucleolar targeting signal of Rex protein of human T-cell leukemia virus type I specifically binds to nucleolar shuttle protein B-23. *The Journal of Biological Chemistry* **268**: 13930-13934.
- Agutter, P. S. and Prochnow, D.** 1994. Nucleocytoplasmic transport. *Biochemical Journal* **300**: 609-618.
- Ainger, K., Avossa, D., Morgan, F., Hill, S. J., Barry, C., Barbarese, E. and Carson, J. H.** 1993. Transport and localization of exogenous myelin basic protein mRNA microinjected into oligodendrocytes. *Journal of Cell Biology* **123**: 431-441.
- Akner, G., Sundqvist, K.-G., Denis, M., Wikström, A.-C. and Gustafsson, J.-A.** 1990. Immunocytochemical localization of glucocorticoid receptor in human gingival fibroblasts and evidence for a colocalization of glucocorticoid receptor with cytoplasmic microtubules. *European Journal of Cell Biology* **53**: 390-401.
- Allison, L. A., North, M. T., Murdoch, K. J., Romaniuk, P. J., Deschamps, S. and le Maire, M.** 1993. Structural requirements of 5S rRNA for nuclear transport, 7S ribonucleoprotein particle assembly, and 60S ribosomal subunit assembly in *Xenopus* oocytes. *Molecular and Cellular Biology* **13**: 6819-6831.
- Allison, L. A., North, M. T. and Neville, L. A.** 1995. Differential binding of oocyte-type and somatic-type 5S rRNA to TFIIA and ribosomal protein L5 in *Xenopus* oocytes: Specialization for storage versus mobilization. *Developmental Biology* **168**: 284-295.
- Allison, L. A., Romaniuk, P. J. and Bakken, A. H.** 1991. RNA-protein interactions of stored 5S RNA with TFIIA and ribosomal protein L5 during *Xenopus* oogenesis. *Developmental Biology* **144**: 129-144.
- Andersen, J., Delihias, N., Hanas, J. S. and Wu, C.-W.** 1984a. 5S RNA structure and interaction with transcription factor A: 1. Ribonuclease probe of the structure of 5S RNA from *Xenopus laevis* oocytes. *Biochemistry* **23**: 5752-5759.
- Andersen, J., Delihias, N., Hanas, J. S. and Wu, C.-W.** 1984b. 5S RNA structure and interaction with transcription factor A: 2. Ribonuclease probe of the 7S particle from *Xenopus laevis* immature oocytes and RNA exchange properties of the 7S particle. *Biochemistry* **23**: 5759-5766.
- Azad, A. A. and Lane, B. G.** 1973. A possible role for 5S RNA as a bridge between ribosomal subunits. *Canadian Journal of Biochemistry* **51**: 1669-1672.

- Azum-Gélade, M.-C., Noaillac-Depeyre, J., Caizergues-Ferrer, M. and Gas, N.** 1994. Cell cycle redistribution of U3 snRNA and fibrillarin: presence in the cytoplasmic nucleolus remnant and in the prenucleolar bodies at telophase. *Journal of Cell Science* **107**: 463-475.
- Bachellerie, J.-P., Michot, B., Nicoloso, M., Balakin, A., Ni, J. and Fournier, M. J.** 1995. Antisense snoRNAs: a family of nucleolar RNAs with long complementarities to rRNA. *Trends in Biochemical Sciences* **20**: 261-264.
- Baeuerle, P. A. and Baltimore, D.** 1988a. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF- κ B transcription factor. *Cell* **53**: 211-217.
- Baeuerle, P. A. and Baltimore, D.** 1988b. I κ B: a specific inhibitor of the NF- κ B transcription factor. *Science* **242**: 540-546.
- Bag, J. and Pramanik, S.** 1987. Attachment of mRNA to the cytoskeletal framework and translational control of gene expression in rat L6 muscle cells. *Biochemistry and Cell Biology* **65**: 565-575.
- Bakken, A., Morgan, G., Sollner-Webb, B., Roan, J., Busby, S. and Reeder, R. H.** 1982. Mapping of transcription initiation and termination signals on *Xenopus laevis* ribosomal DNA. *Proceedings of the National Academy of Sciences USA* **79**: 56-60.
- Bartel, D. P., Zapp, M. L., Green, M. R. and Szostak, J. W.** 1991. HIV-1 Rev regulation involves recognition of non-Watson-Crick base pairs in viral RNA. *Cell* **67**: 529-536.
- Bassel, G. J.** 1993. High resolution distribution of mRNA within the cytoskeleton. *Journal of Cellular Biochemistry* **52**: 127-133.
- Bassel, G. J., Powers, C. M., Taneja, K. L. and Singer, R. H.** 1994. Single mRNAs visualized by ultrastructural in situ hybridization are principally localized at actin filament intersections in fibroblasts. *Journal of Cell Biology* **126**: 863-876.
- Baudin, F. and Romaniuk, P. J.** 1989. A difference in the importance of bulged nucleotides and their parent base pairs in the binding of transcription factor IIIA to *Xenopus* 5S RNA and 5S RNA genes. *Nucleic Acids Research* **17**: 2043-2056.
- Baudin, F., Romaniuk, P. J., Romby, P., Brunel, C., Westhof, E., Ehresmann, B. and Ehresmann, C.** 1991. Involvement of "hinge" nucleotides of *Xenopus laevis* 5S rRNA in the structural organization and in the binding of transcription factor IIIA. *Journal of Molecular Biology* **218**: 69-81.

- Belenguer, P., Baldin, V., Mathieu, C., Prats, H., Bensaid, M., Bouche, G. and Amalric, F. 1989. Protein kinase NII and the regulation of rDNA transcription in mammalian cells. *Nucleic Acids Research* **17**: 6625-6636.
- Bement, W. M. and Capco, D. G. 1990. Transformation of the amphibian oocyte into the egg: structural and biochemical events. *Journal of Electron Microscopy Technique* **16**: 202-234.
- Berezney, R. 1991. The nuclear matrix: a heuristic model for investigating genomic organization and function in the cell nucleus. *Journal of Cellular Biochemistry* **47**: 109-123.
- Bergès, T., Petfalski, E., Tollervey, D. and Hurt, E. C. 1994. Synthetic lethality with fibrillarin identifies NOP77p, a nucleolar protein required for pre-rRNA processing and modification. *The EMBO Journal* **13**: 3136-3148.
- Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., Frigerio, G., Noll, M. and Nüsslein-Volhard, C. 1988. The role of localization of *bicoid* RNA in organising the anterior pattern of the *Drosophila* embryo. *The EMBO Journal* **7**: 1749-1756.
- Biggiogera, M., Fakan, S., Kaufmann, S. H., Black, A., Shaper, J. H. and Busch, H. 1989. Simultaneous immunoelectron microscopic visualization of protein B23 and C23 distribution in the HeLa cell nucleolus. *The Journal of Histochemistry and Cytochemistry* **37**: 1371-1374.
- Bird, R. C. and Sells, B. H. 1986. Cytoskeleton involvement in the distribution of mRNP complexes and small cytoplasmic RNAs. *Biochimica et Biophysica Acta* **868**: 215-225.
- Blanco, J., Millstein, L., Razik, M. A., Dilworth, S., Cote, C. and Gottesfeld, J. 1989. Two TFIIA activities regulate expression of the *Xenopus* 5S RNA gene families. *Genes & Development* **3**: 1602-1612.
- Blobel, G. 1971. Isolation of a 5S RNA-protein complex from mammalian ribosomes. *Proceedings of the National Academy of Sciences USA* **68**: 1881-1885.
- Boelens, W. C., Palacios, I. and Mattaj, I. W. 1995. Nuclear retention of RNA as a mechanism for localization. *RNA* **1**: 273-283.
- Bohmann, K., Ferreira, J. A. and Lamond, A. I. 1995. Mutational analysis of p80 coilin indicates a functional interaction between coiled bodies and the nucleolus. *Journal of Cell Biology* **131**: 817-831.
- Bond, V. C. and Wold, B. 1993. Nucleolar localization of *myc* transcripts. *Molecular and Cellular Biology* **13**: 3221-3230.

- Bonner, W. M.** 1975. Protein migration into nuclei: 1. Frog oocyte nuclei in vivo accumulate microinjected histones, allow entry to small proteins, and exclude large proteins. *Journal of Cell Biology* **64**: 421-430.
- Borer, R. A., Lehner, C. F., Eppenberger, H. M. and Nigg, E. A.** 1989. Major nucleolar proteins shuttle between nucleus and cytoplasm. *Cell* **56**: 379-390.
- Bourgeois, C. A. and Hubert, J.** 1988. Spatial relationship between the nucleolus and the nuclear envelope: structural aspects and functional significance. *International Review of Cytology* **111**: 1-52.
- Bouvet, P. and Wolffe, A. P.** 1994. A role for transcription and FRGY2 in masking maternal mRNA within *Xenopus* oocytes. *Cell* **77**: 931-941.
- Brimacombe, R., Greuer, B., Mitchell, P., Osswald, M., Rinke-Appel, J., Schüler, D. and Stade, K.** 1990. Three-dimensional structure and function of *Escherichia coli* 16S and 23S rRNA as studied by cross-linking techniques. In *The Ribosome: Structure, Function & Evolution* (W. E. Hill, A. Dahlberg, R. A. Garrett, P. B. Moore, D. Schlessinger and J. R. Warner eds) pp 93-106. American Society for Microbiology, Washington, D. C.
- Brown, D. D. and Dawid, I. B.** 1968. Specific gene amplification in oocytes. *Science* **160**: 272-280.
- Brunel, C., Romby, P., Westhof, E., Romaniuk, P. J., Ehresmann, B. and Ehresmann, C.** 1990. Effect of mutations in domain 2 on the structural organization of oocyte 5 S rRNA from *Xenopus laevis*. *Journal of Molecular Biology* **215**: 103-111.
- Cairns, C. and McStay, B.** 1995. Identification and cDNA cloning of a *Xenopus* nucleolar phosphoprotein, xNopp180, that is the homolog of the rat nucleolar protein Nopp140. *Journal of Cell Science* **108**: 3339-3347.
- Caizergues-Ferrer, M., Mariottini, P., Curie, C., Lapeyre, B., Gas, N., Amalric, F. and Amaldi, F.** 1989. Nucleolin from *Xenopus laevis*: cDNA cloning and expression during development. *Genes & Development* **3**: 324-333.
- Callaci, T. P., Cai, G.-Z., Lee, J. C., Daly, T. J. and Wu, C.-W.** 1990. Assembly of *Xenopus* transcription factor III A-5S RNA complex. *Biochemistry* **29**: 4653-4659.
- Capco, D. G. and Bement, W. M.** 1991. Analysis of cellular signalling events, the cytoskeleton and spatial organization of macromolecules during early *Xenopus* development. In *Xenopus laevis: Practical Uses in Cell and Molecular Biology* (B. K. Kay and H. B. Peng eds) pp 249-270. Academic Press, San Diego.

- Cardelli, J., Long, B. and Pitot, H. C. 1976. Direct association of messenger RNA labelled in the presence of fluoroorotate with membranes of the endoplasmic reticulum in rat liver. *Journal of Cell Biology* **70**: 47-58.
- Chan, P. K. and Chan, F. Y. 1995. Nucleophosmin/B23 (NPM) oligomer is a major and stable entity in HeLa cells. *Biochimica et Biophysica Acta* **1262**: 37-42.
- Chan, Y.-L., Lin, A., McNally, J. and Wool, I. G. 1987. The primary structure of rat ribosomal protein L5: a comparison of the sequence of amino acids in the proteins that interact with 5 S rRNA. *The Journal of Biological Chemistry* **262**: 12879-12886.
- Cheng, Y., Dahlberg, J. E. and Lund, E. 1995. Diverse effects of the guanine nucleotide exchange factor RCC1 on RNA transport. *Science* **267**: 1807-1810.
- Cheung, H.-K., Serano, T. L. and Cohen, R. S. 1992. Evidence for a highly selective RNA transport system and its role is establishing the dorsoventral axis of the *Drosophila* egg. *Development* **114**: 653-661.
- Chooi, W. Y. and Leiby, K. R. 1981. An electron microscopic method for localization of ribosomal proteins during transcription of ribosomal DNA: a method for studying protein assembly. *Proceedings of the National Academy of Sciences USA* **78**: 4823-4827.
- Chow, C. S., Hartmann, K. M., Rawlings, S. L., Huber, P. W. and Barton, J. K. 1992. Delineation of structural domains in eukaryotic 5S rRNA with a rhodium probe. *Biochemistry* **31**: 3534-3542.
- Christiansen, J., Brown, R. S., Sproat, B. S. and Garrett, R. A. 1987. *Xenopus* transcription factor IIIA binds primarily at junctions between double helical stems and internal loops in oocyte 5S RNA. *The EMBO Journal* **6**: 452-460.
- Christiansen, J., Douthwaite, S. R., Christensen, A. and Garrett, R. A. 1985. Does unpaired adenosine-66 from helix II of *E. coli* 5S RNA bind to protein L18? *The EMBO Journal* **4**: 1019-1024.
- Clark, M. W. and Lake, J. A. 1984. Unusual rRNA-linked complex of 50S ribosomal subunits isolated from an *Escherichia coli* RNase III mutant. *Journal of Bacteriology* **157**: 971-974.
- Clemens, K. R., Wolf, V., McBryant, S. J., Zhang, P., Liao, X., Wright, P. E. and Gottesfeld, J. M. 1993. Molecular basis for specific recognition of both RNA and DNA by a zinc finger protein. *Science* **260**: 530-533.
- Cochrane, A. W., Perkins, A. and Rosen, C. A. 1990. Identification of sequences important in the nucleolar localization of human immunodeficiency virus Rev: relevance of nucleolar localization to function. *Journal of Virology* **64**: 881-885.

- Colman, A.** 1984. Translation of eukaryotic messenger RNA in *Xenopus* oocytes. In *Transcription and Translation: A Practical Approach* (B. D. Hames and S. J. Higgins eds) pp 271-300. IRL Press, Oxford, Washington, D. C.
- Colombo, R., Benedusi, P. and Valle, G.** 1981. Actin in *Xenopus* development: Indirect immunofluorescence study of actin localization. *Differentiation* **20**: 45-51.
- Coppard, N. J., Poulsen, K., Madsen, H. O., Frydenberg, J. and Clark, B. F. C.** 1991. 42Sp48 in previtellogenic *Xenopus* oocytes is structurally homologous to EF-1 α and may be a stage-specific elongation factor. *Journal of Cell Biology* **112**: 237-243.
- Corsetti, M. T., Levi, G., Lamcia, F., Sanseverino, L., Ferrini, S., Boncinelli, E. and Corte, G.** 1995. Nucleolar localization of three Hox homeoproteins. *Journal of Cell Science* **108**: 187-193.
- Courey, A. J. and Huang, J.-D.** 1995. The establishment and interpretation of transcription factor gradients in the *Drosophila* embryo. *Biochimica et Biophysica Acta* **1261**: 1-18.
- Créancier, L., Prats, H., Zanibellato, C., Amalric, F. and Bugler, B.** 1993. Determination of the functional domains involved in nucleolar targeting of nucleolin. *Molecular Biology of the Cell* **4**: 1239-1250.
- Cripe, L., Morris, E. and Fulton, A. B.** 1993. Vimentin mRNA location changes during muscle development. *Proceedings of the National Academy of Sciences USA* **90**: 2724-2728.
- Dahlberg, A. E.** 1989. The functional role of ribosomal RNA in protein synthesis. *Cell* **57**: 525-529.
- Dalby, B. and Glover, D. M.** 1992. 3' non-translated sequences in *Drosophila* cyclin B transcripts direct posterior pole accumulation late in oogenesis and peri-nuclear association in syncytial embryos. *Development* **115**: 989-997.
- Darnbrough, C. H. and Ford, P. J.** 1981. Identification in *Xenopus laevis* of a class of oocyte-specific proteins bound to messenger RNA. *European Journal of Biochemistry* **113**: 415-424.
- Darsillo, P. and Huber, P. W.** 1991. The use of chemical nucleases to analyze RNA-protein interactions: the TFIIA-5 S rRNA complex. *The Journal of Biological Chemistry* **266**: 21075-21082.
- Davis, I. and Ish-Horowicz, D.** 1991. Apical localization of pair-rule transcripts requires 3' sequences and limits protein diffusion in the *Drosophila* blastoderm embryo. *Cell* **67**: 927-940.

- Dawson, M. and Trapani, J. A.** 1995. The interferon-inducible autoantigen, IFI 16: localization to the nucleolus and identification of a DNA-binding domain. *Biochemical and Biophysical Research Communications* **214**: 152-162.
- Denis, H. and le Maire, M.** 1987. Biochemical research on oogenesis: distribution of tRNA-linked peptides and proteins in previtellogenic oocytes of *Xenopus laevis*. *Biochimie* **69**: 495-509.
- Denis, H. and Wegnez, M.** 1977. Biochemical research on oogenesis: oocytes of *Xenopus laevis* synthesize but do not accumulate 5S RNA of somatic type. *Developmental Biology* **58**: 212-217.
- De Robertis, E., Lienhard, S. and Parisot, R. F.** 1982. Intracellular transport of microinjected 5S and small nuclear RNAs. *Nature* **295**: 572-577.
- Deschamps, S., Viel, A., Garrigos, M., Denis, H. and le Maire, M.** 1992. mRNP4, a major mRNA-binding protein from *Xenopus* oocytes is identical to transcription factor FRG Y2. *The Journal of Biological Chemistry* **267**: 13799-13802.
- Deshmukh, M., Tsay, Y.-F., Paulvich, A. G. and Woolford, J. L.** 1993. Yeast ribosomal protein L1 is required for the stability of newly synthesised 5S rRNA and the assembly of 60S ribosomal subunits. *Molecular and Cellular Biology* **13**: 2835-2845.
- Dickinson, L. A. and Kohwi-Shigematsu, T.** 1995. Nucleolin is a matrix attachment region DNA-binding protein that specifically recognizes a region with high base-unpairing potential. *Molecular and Cellular Biology* **15**: 456-465.
- Dixon, L. K. and Ford, P. J.** 1982a. Persistence of nonribosome bound 5 S RNA in full-grown oocytes of *Xenopus laevis*. *Developmental Biology* **91**: 474-477.
- Dixon, L. K. and Ford, P. J.** 1982b. Regulation of protein synthesis and accumulation during oogenesis in *Xenopus laevis*. *Developmental Biology* **93**: 478-497.
- Dohme, F. and Nierhaus, K. H.** 1976. Role of 5S RNA in assembly and function of the 50S subunit from *Escherichia coli*. *Proceedings of the National Academy of Sciences USA* **73**: 2221-2225.
- Dontsova, O., Tishkov, V., Dokudovskaya, S., Bogdanov, A., Döring, T., Rinke-Appel, J., Thamm, S., Greuer, B. and Brimacombe, R.** 1994. Stem-loop IV of 5S rRNA lies close to the peptidyltransferase center. *Proceedings of the National Academy of Sciences USA* **91**: 4125-4129.
- Draper, D. E.** 1989. How do proteins recognize specific RNA sites? New clues from autogenously regulated ribosomal proteins. *Trends in Biochemical Sciences* **14**: 335-338.

- Draper, D. E. 1995. Protein-RNA recognition. *Annual Review of Biochemistry* **64**: 593-620.
- Dreyer, C., Stick, R. and Hausen, P. 1986. Uptake of oocyte nuclear proteins by nuclei of *Xenopus* embryos. In *Nucleocytoplasmic Transport* (R. Peters and M. Trendelenburg eds) pp 143-157. Springer-Verlag, Berlin Heidelberg.
- Dumbar, T. S., Gentry, G. A. and Olson, M. O. J. 1989. Interaction of nucleolar phosphoprotein B23 with nucleic acids. *Biochemistry* **28**: 9495-9501.
- Dumont, J. N. 1972. Oogenesis in *Xenopus laevis* (Daudin) I. Stages of oocyte development in laboratory maintained animals. *Journal of Morphology* **136**: 153-180.
- Dundr, M., Leno, G. H., Hammarskjöld, M.-L., Rekosh, D., Helga-Maria, C. and Olson, M. O. J. 1995. The roles of nucleolar structure and function in the subcellular location of the HIV-1 rev protein. *Journal of Cell Science* **108**: 2811-2823.
- Dundr, M. and Raška, I. 1993. Nonisotopic ultrastructural mapping of transcription sites within the nucleolus. *Experimental Cell Research* **208**: 275-281.
- Egebjerg, J., Christiansen, J., Brown, R. S., Larsen, N. and Garrett, R. A. 1989. Protein L18 binds primarily at the junctions of helix II and internal loops A and B in *Escherichia coli* 5S RNA. *Journal of Molecular Biology* **206**: 651-668.
- Eichler, D. C. and Craig, N. 1994. Processing of eukaryotic ribosomal RNA. *Progress in Nucleic Acid Research and Molecular Biology* **49**: 197-239.
- Elisha, Z., Havin, L., Ringel, I. and Yisraeli, J. K. 1995. Vg1 RNA binding protein mediates the association of Vg1 RNA with microtubules in *Xenopus* oocytes. *The EMBO Journal* **14**: 5109-5114.
- Engelke, D. R., Ng, S.-Y., Shastry, B. S. and Roeder, G. 1980. Specific interaction of a purified transcription factor with an internal control region of 5S RNA genes. *Cell* **19**: 717-728.
- Erdmann, V. A. 1976. Structure and function of 5S and 5.8S RNA. *Progress in Nucleic Acid Research and Molecular Biology* **18**: 45-90.
- Evans, W. H. and Graham, J. M. 1989. *Membrane Structure and Function*. IRL Press, Oxford.
- Evstafieva, A. G., Shatsky, I. N., Bogdanov, A. A. and Vasiliev, V. D. 1985. Topography of RNA in the ribosome: location of the 5 S RNA residues A₃₉ and U₄₀ on the central protuberance of the 50 S subunit. *FEBS Letters* **185**: 57-62.

- Fabijanski, S. and Pellegrini, M.** 1981. Identification of proteins at the peptidyl-tRNA binding site of rat liver ribosomes. *Molecular and General Genetics* **184**: 551-556.
- Fankhauser, C., Izaurralde, E., Adachi, Y., Wingfield, P. and Laemmli, U. K.** 1991. Specific complex of human immunodeficiency virus type 1 Rev and nucleolar B23 proteins: dissociation by the Rev response element. *Molecular and Cellular Biology* **11**: 2567-2575.
- Featherstone, C., Darby, M. K. and Gerace, L.** 1988. A monoclonal antibody against the nuclear pore complex inhibits nucleocytoplasmic transport of protein and RNA in vivo. *Journal of Cell Biology* **107**: 1289-1297.
- Felber, B. K., Hadzopoulou-Cladaras, M., Cladaras, C., Copeland, T. and Pavlakis, G. N.** 1989. rev protein of human immunodeficiency virus type 1 affects the stability and transport of the viral mRNA. *Proceedings of the National Academy of Sciences USA* **86**: 1495-1499.
- Feldherr, C. M. and Akin, D.** 1994. Role of nuclear trafficking in regulating cellular activity. *International Review of Cytology* **151**: 183-228.
- Ferrandon, D., Elphick, L., Nüsslein-Volhard, C. and St. Johnston, D.** 1994. Stauf protein associates with the 3' UTR of *bicoid* mRNA to form particles that move in a microtubule-dependent manner. *Cell* **79**: 1221-1232.
- Feuerstein, N. and Mond, J. J.** 1987. "Numatrin," a nuclear matrix protein associated with induction of proliferation in B lymphocytes. *The Journal of Biological Chemistry* **262**: 11389-11397.
- Finch, R. A., Revankar, G. R. and Chan, P. K.** 1993. Nucleolar localization of nucleophosmin/B23 requires GTP. *The Journal of Biological Chemistry* **268**: 5823-5827.
- Fischer, D., Hock, R. and Scheer, U.** 1993. DNA topoisomerase II is not detectable on lampbrush chromosomes but enriched in the amplified nucleoli of *Xenopus* oocytes. *Experimental Cell Research* **209**: 255-260.
- Fischer, D., Weisenberger, D. and Scheer, U.** 1991a. Assigning functions to nucleolar structures. *Chromosoma* **101**: 133-140.
- Fischer, U., Darzynkiewicz, E., Tahara, S. M., Dathan, N. A., Lührmann, R. and Mattaj, I. W.** 1991b. Diversity in the signals required for nuclear accumulation of U snRNPs and variety in the pathways of nuclear transport. *Journal of Cell Biology* **113**: 705-714.
- Fischer, U. and Lührmann, R.** 1990. An essential signaling role for the m₃G cap in the transport of U1 snRNP to the nucleus. *Science* **249**: 786-790.

- Ford, P. J. 1971. Non-coordinated accumulation and synthesis of 5S ribonucleic acid by ovaries of *Xenopus laevis*. *Nature* **233**: 561-564.
- Ford, P. J. and Southern, E. M. 1973. Different sequences for 5S RNA in kidney cells and ovaries of *Xenopus laevis*. *Nature New Biology* **241**: 7-12.
- Forristall, C., Pondel, M., Chen, L. and King, M. L. 1995. Patterns of localization and cytoskeletal association of two vegetally localized RNAs, *Vgl* and *Xcat-2*. *Development* **121**: 201-208.
- Frank, J., Verschoor, A., Radermacher, M. and Wagenknecht, T. 1990. Morphologies of eubacterial and eucaryotic ribosomes as determined by three-dimensional electron microscopy. In *The Ribosome: Structure, Function & Evolution* (W. E. Hill, A. Dahlberg, R. A. Garrett, P. B. Moore, D. Schlessinger and J. R. Warner eds) pp 107-113. American Society for Microbiology, Washington, D. C.
- Gait, M. J. and Karn, J. 1993. RNA recognition by the human immunodeficiency virus Tat and Rev proteins. *Trends in Biochemical Sciences* **18**: 255-259.
- Gall, J. G. 1968. Differential synthesis of the genes for ribosomal RNA during amphibian oogenesis. *Proceedings of the National Academy of Sciences USA* **60**: 553-560.
- Gall, L., Picheral, B. and Gounon, P. 1983. Cytochemical evidence for the presence of intermediate filaments and microfilaments in the egg of *Xenopus laevis*. *Biology of the Cell* **47**: 331-342.
- Gamberi, C., Contreas, G., Romanelli, M. G. and Morandi, C. 1994. Analysis of the yeast *NSR1* gene and protein domain comparison between Nsr1 and human hnRNP type A1. *Gene* **148**: 59-66.
- Gard, D. L. 1991. Organisation, nucleation, and acetylation of microtubules in *Xenopus laevis* oocytes: A study by confocal immunofluorescence microscopy. *Developmental Biology* **143**: 346-362.
- Gard, D. L. 1995. Axis formation during amphibian oogenesis: reevaluating the role of the cytoskeleton. *Current Topics in Developmental Biology* **30**: 215-252.
- Garner, C. C., Tucker, R. P. and Matus, A. 1988. Selective localization of messenger RNA for cytoskeletal protein MAP2 in dendrites. *Nature* **336**: 674-677.
- Garrett, R. A., Douthwaite, S. and Noller, H. F. 1981. Structure and role of 5S RNA-protein complexes in protein biosynthesis. *Trends in Biochemical Sciences* **6**: 137-139.
- Gavis, E. R. and Lehmann, R. 1992. Localization of *nanos* RNA controls embryonic polarity. *Cell* **71**: 301-313.

- Geisler, R., Bergmann, A., Hiromi, Y. and Nüsslein-Volhard, C. 1992. *cactus*, a gene involved in dorsoventral pattern formation of *Drosophila*, is related to the I κ B gene family of vertebrates. *Cell* 71: 613-621.
- Gerbi, S. A., Savino, R., Stebbins-Boaz, B., Jeppesen, C. and Rivera-León, R. 1990. A role for U3 small nuclear ribonucleoprotein in the nucleolus? In *The Ribosome: Structure, Function, & Evolution* (W. E. Hill, A. Dahlberg, R. A. Garrett, P. B. Moore, D. Schlessinger and J. R. Warner eds) pp 452-469. American Society for Microbiology, Washington, D. C.
- Ghisolfi, L., Joseph, G., Erard, M., Escoubas, J.-M., Mathieu, C. and Amalric, F. 1990. Nucleolin - pre-rRNA interactions and preribosome assembly. *Molecular Biology Reports* 14: 113-114.
- Ghisolfi, L., Kharrat, A., Joseph, G., Amalric, F. and Erard, M. 1992. Concerted activities of the RNA recognition and the glycine-rich C-terminal domains of nucleolin are required for efficient complex formation with pre-ribosomal RNA. *FEBS Letters* 209: 541-548.
- Ginsberg, A. M., King, B. O. and Roeder, R. G. 1984. *Xenopus* 5S gene transcription factor, TFIIIA: characterization of a cDNA clone and measurement of RNA levels throughout development. *Cell* 39: 479-489.
- Godsave, S. F., Anderton, B. H., Heasman, J. and Wylie, C. C. 1984a. Oocytes and early embryos of *Xenopus laevis* contain intermediate filaments which react with anti-mammalian vimentin antibodies. *Journal of Embryology and Experimental Morphology* 83: 169-187.
- Godsave, S. F., Wylie, C. C., Lane, E. B. and Anderton, B. H. 1984b. Intermediate filaments in the *Xenopus* oocyte: the appearance and distribution of cytokeratin-containing filaments. *Journal of Embryology and Experimental Morphology* 83: 157-167.
- Görlich, D., Vogel, F., Mills, A. D., Hartmann, E. and Laskey, R. A. 1995. Distinct functions for the two importin subunits in nuclear protein import. *Nature* 377: 246-248.
- Goessens, G. 1984. Nucleolar structure. *International Review of Cytology* 87: 107-158.
- Goldfarb, D. S. 1988. Karyophilic peptides: applications to the study of nuclear transport. *Cell Biology International Reports* 12: 809-832.
- Gottlieb, E. and Steitz, J. A. 1989. Function of the mammalian La protein: evidence for its action in transcription termination by RNA polymerase III. *The EMBO Journal* 8: 851-861.

- Granboulan, N. and Granboulan, P. 1965. Cytochimie ultrastructurale du nucleole. *Experimental Cell Research* **38**: 604-619.
- Grosfeld, H., Soreq, H. and Littauer, U. Z. 1977. Membrane associated cytoplasmic mRNA in *Artemia salina*; functional and physical changes during development. *Nucleic Acids Research* **4**: 2109-2121.
- Grossi de Sa, M.-F., Martins de Sa, C. M., Harper, F., Olink-Coux, M., Huesca, M. and Scherrer, K. 1988. The association of prosomes with some of the intermediate filament networks of the animal cell. *Journal of Cell Biology* **107**: 1517-1530.
- Grummt, F., Grummt, I. and Erdmann, V. A. 1974. ATPase and GTPase activities isolated from rat liver ribosomes. *European Journal of Biochemistry* **43**: 343-348.
- Guddat, U., Bakken, A. H. and Pieler, T. 1990. Protein-mediated nuclear export of RNA: 5S rRNA containing small RNPs in *Xenopus* oocytes. *Cell* **60**: 619-628.
- Gulli, M.-P., Girard, J.-P., Zabetakis, D., Lapeyre, B., Mélése, T. and Caizergues-Ferrer, M. 1995. gar2 is a nucleolar protein from *Schizosaccharomyces pombe* required for 18S rRNA and 40S ribosomal subunit accumulation. *Nucleic Acids Research* **23**: 1912-1918.
- Gururajan, R., Mathews, L., Longo, F. J. and Weeks, D. L. 1994. An3 mRNA encodes an RNA helicase that colocalizes with nucleoli in *Xenopus* oocytes in a stage specific manner. *Proceedings of the National Academy of Sciences USA* **91**: 2056-2060.
- Hadjiolov, A. A. 1985. *The Nucleolus and Ribosome Biogenesis*. Springer-Verlag, Vienna, New York.
- Hamm, J., Darzynkiewicz, E., Tahara, S. M. and Mattaj, I. W. 1990. The trimethylguanosine cap structure of U1 snRNA is a component of a bipartite nuclear targeting signal. *Cell* **62**: 569-577.
- Hamm, J., Kazmaier, M. and Mattaj, I. W. 1987. *In vitro* assembly of U1 snRNPs. *The EMBO Journal* **6**: 3479-3485.
- Harlow, E. and Lane, D. 1988. *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.
- Hauber, J., Perkins, A., Heimer, E. P. and Cullen, B. R. 1987. Trans-activation of human immunodeficiency virus gene expression is mediated by nuclear events. *Proceedings of the National Academy of Sciences USA* **84**: 6364-6368.
- Hausen, P. and Riebesell, M. 1991. *The Early Development of Xenopus laevis: An Atlas of the Histology*. Springer-Verlag, Berlin.

- Heine, M. A., Rankin, M. L. and DiMario, P. J.** 1993. The gly/arg-rich (GAR) domain of *Xenopus* nucleolin facilitates in vitro nucleic acid binding and in vivo nucleolar localization. *Molecular Biology of the Cell* **4**: 1189-1204.
- Henderson, B. R., Menotti, E., Bonnard, C. and Kühn, L. C.** 1994. Optimal sequence and structure of iron-responsive elements: Selection of RNA stem-loops with high affinity for iron regulatory factor. *The Journal of Biological Chemistry* **269**: 17481-17489.
- Hernandez-Verdun, D.** 1991. The nucleolus today. *Journal of Cell Science* **99**: 465-471.
- Herrera, J. E., Savkur, R. and Olson, M. O. J.** 1995. The ribonuclease activity of nucleolar protein B23. *Nucleic Acids Research* **23**: 3974-3979.
- Hesketh, J., Campbell, G., Piechaczyk, M. and Blanchard, J.-M.** 1994. Targeting of c-myc and β -globin coding sequences to cytoskeletal-bound polysomes by c-myc 3' untranslated region. *Biochemical Journal* **298**: 143-148.
- Hirano, K., Ito, M. and Hartshorne, D. J.** 1995. Interaction of the ribosomal protein, L5, with protein phosphatase type 1. *The Journal of Biological Chemistry* **270**: 19786-19790.
- Holmberg, L., Melander, Y. and Nygård, O.** 1992. Ribosome-bound eukaryotic elongation factor 2 protects 5 S rRNA from modification. *The Journal of Biological Chemistry* **267**: 21906-21910.
- Honda, B. M. and Roeder, R. G.** 1980. Association of 5S gene transcription factor with 5S RNA and altered levels of the factor during cell differentiation. *Cell* **22**: 119-126.
- Hozák, P.** 1995. Catching RNA polymerase I *in flagranti*: ribosomal genes are transcribed in the dense fibrillar component of the nucleolus. *Experimental Cell Research* **216**: 285-289.
- Hozák, P., Cook, P. R., Schöfer, C., Mosgöller, W. and Wachtler, F.** 1994. Site of transcription of ribosomal RNA and intranucleolar structure in HeLa cells. *Journal of Cell Science* **107**: 639-648.
- Huber, P. W. and Wool, I. G.** 1984. Nuclease protection analysis of ribonucleoprotein complexes: use of the cytotoxic ribonuclease α -sarcin to determine the binding sites for *Escherichia coli* ribosomal proteins L5, L18 and L25 on 5S rRNA. *Proceedings of the National Academy of Sciences USA* **81**: 322-326.
- Huber, P. W. and Wool, I. G.** 1986a. Identification of the binding site on 5S RNA for the transcription factor IIIA: proposed structure of a common binding site on 5S rRNA and on the gene. *Proceedings of the National Academy of Sciences USA* **83**: 1593-1597.

- Huber, P. W. and Wool, I. R. 1986b. Use of the cytotoxic nuclease α -sarcin to identify the binding site on eukaryotic 5 S ribosomal ribonucleic acid for the ribosomal protein L5. *The Journal of Biological Chemistry* **261**: 3002-3005.
- Hügler, B., Scheer, U. and Franke, W. W. 1985. Ribocharin: a nuclear M_r 40,000 protein specific to precursor particles of the large ribosomal subunit. *Cell* **41**: 615-627.
- Hunt, T. 1989. Cytoplasmic anchoring proteins and the control of nuclear localization. *Cell* **59**: 949-951.
- Inouye, C. J. and Seto, E. 1994. Relief of YY1-induced transcriptional repression by protein-protein interaction with the nucleolar phosphoprotein B23. *The Journal of Biological Chemistry* **269**: 6506-6510.
- Isoda, K. and Nüsslein-Volhard, C. 1994. Disulphide cross-linking in crude embryonic lysates reveals three complexes of the *Drosophila* morphogen dorsal and its inhibitor cactus. *Proceedings of the National Academy of Sciences USA* **91**: 5350-5354.
- Jackson, S. P. and Tijan, R. 1988. O-glycosylation of eukaryotic transcription factors: implications for mechanisms of transcriptional regulation. *Cell* **55**: 125-133.
- Jaffrey, S. R., Haile, D. J., Klausner, R. D. and Harford, J. B. 1993. The interaction between the iron-responsive element binding protein and its cognate RNA is highly dependent upon both RNA sequence and structure. *Nucleic Acids Research* **19**: 5173-5180.
- Jakob, R. 1993. Nucleolar accumulation of core protein in cells naturally infected with Semliki Forest virus. *Virus Research* **30**: 145-160.
- Jakob, R. 1994. Nucleolar accumulation of Semliki Forest virus nucleocapsid C protein: influence of metabolic status, cytoskeleton and receptors. *Journal of Medical Microbiology* **40**: 389-392.
- Jessus, C., Thibier, C. and Ozon, R. 1987. Levels of microtubules during meiotic maturation of the *Xenopus* oocyte. *Journal of Cell Science* **87**: 705-712.
- Jiménez-García, L. F., Segura-Valdez, M. de L., Ochs, R. L., Rothblum, L. I., Hannan, R. and Spector, D. L. 1994. Nucleologenesis: U3 snRNA-containing prenucleolar bodies move to sites of active pre-rRNA transcription after mitosis. *Molecular Biology of the Cell* **5**: 955-966.
- Joho, K. E., Darby, M. K., Crawford, E. T. and Brown, D. D. 1990. A finger protein structurally similar to TFIIA that binds exclusively to 5S RNA in *Xenopus*. *Cell* **61**: 293-300.

- Kalderon, D., Richardson, W. D., Markham, A. F. and Smith, A. E. 1984a. Sequence requirements for nuclear location of Simian virus 40 large-T antigen. *Nature* **311**: 33-38.
- Kalderon, D., Roberts, B. L., Richardson, W. D. and Smith, A. E. 1984b. A short amino acid sequence able to specify nuclear location. *Cell* **39**: 499-509.
- Kedersha, N. L. and Rome, L. H. 1990. Vaults: Large cytoplasmic RNP's that associate with cytoskeletal elements. *Molecular Biology Reports* **14**: 121-122.
- Kelly, J. M. and Cox, R. A. 1982. The nucleotide sequence at the 3'-end of *Neurospora crassa* 18S-rRNA and studies on the interaction with 5S-rRNA. *Nucleic Acids Research* **10**: 6733-6745.
- Kenmochi, N., Maeda, N. and Tanaka, T. 1991. The primary structure of chicken ribosomal protein L5. *Biochimica et Biophysica Acta* **1088**: 445-447.
- Kenmochi, N. and Ogata, K. 1989. The 5S RNA binding protein of *Artemia salina* ribosomes: identification and immunological comparison with that of rat liver. *Zoological Science* **6**: 295-301.
- Kessel, R. G. 1992. Annulate lamellae: A last frontier in cellular organelles. *International Review of Cytology* **133**: 43-120.
- Kiefer, P. and Dickson, C. 1995. Nucleolar association of fibroblast growth factor 3 via specific sequence motifs has inhibitory effects on cell growth. *Molecular and Cellular Biology* **15**: 4364-4374.
- Kim, S. H., Darby, M. K., Joho, K. E. and Brown, D. D. 1990. The characterization of the TFIIIA synthesised in somatic cells of *Xenopus laevis*. *Genes & Development* **4**: 1602-1610.
- Kim-Ha, J., Smith, J. L. and Macdonald, P. M. 1991. *oskar* mRNA is localized to the posterior pole of the Drosophila oocyte. *Cell* **66**: 23-35.
- Kim-Ha, J., Webster, P. J., Smith, J. L. and Macdonald, P. M. 1993. Multiple RNA regulatory elements mediate distinct steps in localization of *oskar* mRNA. *Development* **119**: 169-178.
- King, M. L. and Barklis, E. 1985. Regional distribution of maternal messenger RNA in the amphibian oocyte. *Developmental Biology* **112**: 203-212.
- Kislauskis, E. H., Li, Z., Singer, R. H. and Taneja, K. L. 1993. Isoform-specific 3'-untranslated sequences sort α -cardiac and β -cytoplasmic actin messenger RNAs to different cytoplasmic compartments. *Journal of Cell Biology* **123**: 165-172.

- Kislauskis, E. H., Zhu, X. and Singer, R. H. 1994. Sequences responsible for intracellular localization of β -actin messenger RNA also affect cell phenotype. *Journal of Cell Biology* **127**: 441-451.
- Kloc, M. and Etkin, L. D. 1994. Delocalization of Vg1 mRNA from the vegetal cortex in *Xenopus* oocytes after destruction of Xlsirt RNA. *Science* **265**: 1101-1103.
- Kloc, M. and Etkin, L. D. 1995. Two distinct pathways for the localization of RNAs at the vegetal cortex in *Xenopus* oocytes. *Development* **121**: 287-297.
- Kloc, M., Spohr, G. and Etkin, L. D. 1993. Translocation of repetitive RNA sequences with the germ plasm in *Xenopus* oocytes. *Science* **262**: 1712-1714.
- Klymkowsky, M. W., Maynell, L. A. and Nislow, C. 1991. Cytokeratin phosphorylation, cytotkeratin filament severing and the solubilization of the maternal mRNA Vg1. *Journal of Cell Biology* **114**: 787-797.
- Klymkowsky, M. W., Maynell, L. A. and Polson, A. G. 1987. Polar asymmetry in the organization of the cortical cytotkeratin system of *Xenopus laevis* oocytes and embryos. *Development* **100**: 543-557.
- Knight, E. J. and Darnell, J. E. 1967. Distribution of 5s RNA in HeLa cells. *Journal of Molecular Biology* **28**: 491-502.
- Ku, M. and Melton, D. A. 1993. *Xwnt-11*: a maternally expressed *Xenopus wnt* gene. *Development* **119**: 1161-1173.
- Kubota, S., Siomi, H., Satoh, T., Endo, S.-I., Maki, M. and Hatanaka, M. 1989. Functional similarity of HIV-I rev and HTLV-I rex proteins: identification of a new nucleolar-targeting signal in rev protein. *Biochemical and Biophysical Research Communications* **162**: 963-970.
- Kumar, A. and Warner, J. R. 1972. Characterization of ribosomal precursor particles from HeLa cell nucleoli. *Journal of Molecular Biology* **63**: 233-246.
- Lagaye, S., Barque, J.-P., le Maire, M., Denis, H. and Larsen, C.-J. 1988. Characterization by human antibodies of two HeLa cell proteins which are related to *Xenopus laevis* transcription factor IIIA. *Nucleic Acids Research* **16**: 2473-2487.
- Lande, M. A., Adesnik, M., Sumida, M., Tashiro, J. and Sabatini, D. D. 1975. Direct association of messenger RNA with microsomal membranes in human diploid fibroblasts. *Journal of Cell Biology* **65**: 513-528.
- Lantz, V. and Schedl, P. 1994. Multiple *cis*-acting targeting sequences are required for *orb* mRNA localization during *Drosophila* oogenesis. *Molecular and Cellular Biology* **14**: 2235-2242.

- Lapeyre, B., Bourbon, H. and Amalric, F.** 1987. Nucleolin, the major nucleolar protein of growing eukaryotic cells: an unusual protein structure revealed by nucleotide sequence. *Proceedings of the National Academy of Sciences USA* **84**: 1472-1476.
- Lawrence, J. B. and Singer, R. H.** 1986. Intracellular localization of messenger RNAs for cytoskeletal proteins. *Cell* **45**: 407-415.
- Le, S.-Y., Pattabiraman, N. and Maizel, J. V. J.** 1994. RNA tertiary structure of the HIV RRE domain II containing non-Watson-Crick base pairs GG and GA: molecular modelling studies. *Nucleic Acids Research* **22**: 3966-3976.
- Leal de Stevenson, I., Romby, P., Baudin, F., Brunel, C., Westhof, E., Ehresmann, C., Ehresmann, B. and Romaniuk, P. J.** 1991. Structural studies on site-directed mutants of domain 3 of *Xenopus laevis* oocyte 5 S ribosomal RNA. *Journal of Molecular Biology* **219**: 243-255.
- Lee, W.-C., Xue, Z. and Mélése, T.** 1991. The *NSR1* gene encodes a protein that specifically binds nuclear localization sequences and has two RNA recognition motifs. *Journal of Cell Biology* **113**: 1-12.
- Lee, W.-C., Zabetakis, D. and Mélése, T.** 1992. NSR1 is required for pre-rRNA processing and for the proper maintenance of steady-state levels of ribosomal subunits. *Molecular and Cellular Biology* **12**: 3865-3871.
- Leibowitz, R. D., Weinberg, R. A. and Penman, S.** 1973. Unusual metabolism of 5 S RNA in HeLa cells. *Journal of Molecular Biology* **73**: 139-144.
- le Maire, M. and Denis, H.** 1987. Biochemical research on oogenesis: Binding of tRNA to the nucleoprotein particles of *Xenopus laevis* previtellogenic oocytes. *The Journal of Biological Chemistry* **262**: 654-659.
- Lenk, R., Ransom, L., Kaufmann, Y. and Penman, S.** 1977. A cytoskeletal structure with associated polyribosomes obtained from HeLa cells. *Cell* **10**: 67-78.
- Li, X., Shou, W., Kloc, M., Reddy, B. A. and Etkin, L. D.** 1994. Cytoplasmic retention of *Xenopus* nuclear factor 7 before the mid blastula transition uses a unique anchoring mechanism involving a retention domain and several phosphorylation sites. *Journal of Cell Biology* **124**: 7-17.
- Lilley, D. M. J.** 1995. Kinking of DNA and RNA by base bulges. *Proceedings of the National Academy of Sciences USA* **92**: 7140-7142.
- Linnen, J. M., Bailey, C. P. and Weeks, D. L.** 1993. Two related mRNAs from *Xenopus laevis* encode ubiquitin-like fusion proteins. *Gene* **128**: 181-188.

- Lischwe, M. A., Ochs, R. L., Reddy, R., Cook, R. G., Yeoman, L. C., Tan, E. M., Reichlin, M. and Busch, H. 1985. Purification and partial characterization of a nucleolar scleroderma antigen ($M_r = 34,000$; pI, 8.5) rich in N^G, N^G -dimethylarginine. *The Journal of Biological Chemistry* **260**: 14304-14310.
- Litman, P., Barg, J. and Ginzburg, I. 1994. Microtubules are involved in the localization of tau mRNA in primary neuronal cell cultures. *Neuron* **13**: 1463-1474.
- Liu, Y.-C. and Chou, Y.-C. 1990. Formaldehyde in formaldehyde/agarose gel may be eliminated without affecting the electrophoretic separation of RNA molecules. *BioTechniques* **9**: 558-560.
- Lo, A. C. and Nazar, R. N. 1982. Topography of 5.8S rRNA in rat liver ribosomes. *The Journal of Biological Chemistry* **257**: 3516-3524.
- Macdonald, P. M. 1990. *bicoid* mRNA localisation signal: phylogenetic conservation of function and RNA secondary structure. *Development* **110**: 161-171.
- Macdonald, P. M., Kerr, K., Smith, J. L. and Leask, A. 1993. RNA regulatory element BLE1 directs the early steps of *bicoid* mRNA localization. *Development* **118**: 1233-1243.
- Macdonald, P. M. and Struhl, G. 1988. *Cis*-acting sequences responsible for anterior localization of *bicoid* mRNA in *Drosophila*. *Nature* **336**: 595-598.
- Maden, B. E. 1990. The numerous modified nucleotides in eukaryotic ribosomal RNA. *Progress in Nucleic Acid Research and Molecular Biology* **39**: 241-303.
- Maeda, Y., Hisatake, K., Kondo, T., Hanada, K.-I., Song, C.-Z., Nishimura, T. and Muramatsu, M. 1992. Mouse rRNA gene transcription factor mUBF requires both HMG-box1 and an acidic tail for nucleolar accumulation: molecular analysis of the nucleolar targeting mechanism. *The EMBO Journal* **11**: 3695-3704.
- Malatesta, M., Zancanaro, C., Martin, T. E., Chan, E. K. L., Amalric, F., Lührmann, R., Vogel, P. and Fakan, S. 1994. Is the coiled body involved in nucleolar functions? *Experimental Cell Research* **211**: 415-419.
- Mao, X. and Darby, M. K. 1993. A position-dependent transcription-activating domain in TFIIIA. *Molecular and Cellular Biology* **13**: 7496-7506.
- Marechal, V., Elenbaas, B., Piette, J., Nicolas, J.-C. and Levine, A. J. 1994. The ribosomal L5 protein is associated with mdm-2 and mdm-2-p53 complexes. *Molecular and Cellular Biology* **14**: 7414-7420.

- Mattaj, I. W.** 1986. The role of RNA-protein interactions in intracellular targeting. In *Nucleocytoplasmic Transport* (R. Peters and M. Trendelenburg eds) pp 275-285. Springer-Verlag, Berlin Heidelberg.
- Mattaj, I. W., Lienhard, S., Zeller, R. and DeRobertis, E. M.** 1983. Nuclear exclusion of transcription factor IIIA and the 42S particle transfer RNA-binding protein in *Xenopus* oocytes: A possible mechanism for gene control. *Journal of Cell Biology* **97**: 1261-1265.
- Maxwell, E. S. and Fournier, M. J.** 1995. The small nucleolar RNAs. *Annual Review of Biochemistry* **64**: 897-934.
- McBryant, S. J., Veldhoen, N., Gedulin, B., Leresche, A., Foster, M. P., Wright, P. E., Romaniuk, P. J. and Gottesfeld, J. M.** 1995. Interaction of the RNA binding fingers of *Xenopus* transcription factor IIIA with specific regions of 5 S ribosomal RNA. *Journal of Molecular Biology* **248**: 44-57.
- McKnight, S. L., Hipskind, R. A. and Reeder, R.** 1980. Ultrastructural analysis of ribosomal gene transcription *in vitro*. *The Journal of Biological Chemistry* **255**: 9707-9711.
- Mears, W. E., Lam, V. and Rice, S. A.** 1995. identification of nuclear and nucleolar localization signals in the herpes simplex virus regulatory protein ICP27. *Journal of Virology* **69**: 935-947.
- Mehlin, H., Daneholt, B. and Skoglund, U.** 1992. Translocation of a specific premessenger ribonucleoprotein particle through the nuclear pore studied with electron microscope tomography. *Cell* **69**: 605-613.
- Meier, N., Göringer, H. U., Klevers, B., Scheibe, U., Eberle, J., Szymkowiak, C., Zacharias, M. and Wagner, R.** 1986. The importance of individual nucleotides for the structure and function of rRNA molecules in *E. coli*. *FEBS Letters* **204**: 89-95.
- Meier, U. T. and Blobel, G.** 1990. A nuclear localization signal binding protein in the nucleolus. *Journal of Cell Biology* **111**: 2235-2245.
- Meier, U. T. and Blobel, G.** 1992. Nopp140 shuttles on tracks between nucleolus and cytoplasm. *Cell* **70**: 127-138.
- Meier, U. T. and Blobel, G.** 1994. NAP57, a mammalian nucleolar protein with a putative homolog in yeast and bacteria. *Journal of Cell Biology* **127**: 1505-1514.
- Mélèse, T. and Xue, Z.** 1995. The nucleolus: an organelle formed by the act of building a ribosome. *Current Opinion in Cell Biology* **7**: 319-324.

- Melton, D. A.** 1987. Translocation of a localized maternal mRNA to the vegetal pole of *Xenopus* oocytes. *Nature* **328**: 80-82.
- Merriam, R. W. and Clark, T. G.** 1978. Actin in *Xenopus* oocytes. II. Intracellular distribution and polymerizability. *Journal of Cell Biology* **77**: 439-447.
- Meßmer, B. and Dreyer, C.** 1993. Requirements for nuclear translocation and nucleolar accumulation of nucleolin of *Xenopus laevis*. *European Journal of Cell Biology* **61**: 369-382.
- Metspalu, A., Toots, I., Saarma, M. and Villems, R.** 1980. The ternary complex consisting of rat liver ribosomal 5 S RNA, 5.8 S RNA and protein L5. *FEBS Letters* **119**: 81-84.
- Meyer, B. E. and Malim, M. H.** 1994. The HIV-1 Rev *trans*-activator shuttles between the nucleus and the cytoplasm. *Genes & Development* **8**: 1538-1547.
- Milcarek, C. and Penman, S.** 1974. Membrane-bound polyribosomes in HeLa cells: Association of polyadenylic acid with membranes. *Journal of Molecular Biology* **89**: 327-338.
- Miller, J., McLachlan, A. D. and Klug, A.** 1985. Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus* oocytes. *The EMBO Journal* **4**: 1609-1614.
- Miller, L.** 1974. Metabolism of 5S RNA in the absence of ribosome production. *Cell* **3**: 275-281.
- Miller, M., Reddy, B. A., Kloc, M., Li, X. X., Dreyer, C. and Etkin, L. D.** 1991. The nuclear-cytoplasmic distribution of the *Xenopus* nuclear factor, xnf7, coincides with its state of phosphorylation during early development. *Development* **113**: 569-575.
- Moore, M. S. and Blobel, G.** 1994. A G protein involved in nucleocytoplasmic transport: the role of Ran. *Trends in Biochemical Sciences* **19**: 211-216.
- Moroianu, J. and Riordan, J. F.** 1994. Identification of the nucleolar targeting signal of human angiogenin. *Biochemical and Biophysical Research Communications* **203**: 1765-1772.
- Mosquera, L., Foristall, C., Zhou, Y. and King, M. L.** 1993. A mRNA localized to the vegetal cortex of *Xenopus* oocytes encodes a protein with a *nanos*-like zinc finger domain. *Development* **117**: 377-386.
- Mougey, E. B., Pape, L. K. and Sollner-Webb, B.** 1993. A U3 small nuclear ribonucleoprotein-requiring processing event in the 5' external transcribed spacer of *Xenopus* precursor rRNA. *Molecular and Cellular Biology* **13**: 5990-5998.

- Mowry, K. L. and Melton, D. A. 1992. Vegetal messenger RNA localization directed by a 340-nt sequence element in *Xenopus* oocytes. *Science* **255**: 991-994.
- Murray, M. T., Schiller, D. L. and Franke, W. W. 1992. Sequence analysis of cytoplasmic mRNA-binding proteins of *Xenopus* oocytes identifies a family of RNA-binding proteins. *Proceedings of the National Academy of Sciences USA* **89**: 11-15.
- Murti, K. and Goorha, R. 1989. Synthesis of frog virus 3 proteins occurs on intermediate filament-bound polyribosomes. *Biology of the Cell* **65**: 205-214.
- Nazar, R. N. 1979. The ribosomal protein binding site in *Saccharomyces cerevisiae* ribosomal 5 S RNA: A conserved protein binding site in 5 S RNA. *The Journal of Biological Chemistry* **254**: 7724-7729.
- Nazar, R. N. 1991. Higher order structure of the ribosomal 5 S RNA. *The Journal of Biological Chemistry* **266**: 4562-4567.
- Nazar, R. N. and Wildeman, A. G. 1983. Three helical domains form a protein binding site in the 5S RNA-protein complex from eukaryotic ribosomes. *Nucleic Acids Research* **11**: 3155-3168.
- Nazar, R. N., Yaguchi, M. and Willick, G. E. 1982. The 5S RNA-protein complex from yeast: a model for the evolution and structure of the eukaryotic ribosome. *Canadian Journal of Biochemistry* **60**: 490-496.
- Neenhold, H. R. and Rana, T. M. 1995. Major groove opening at the HIV-1 Tat binding site of TAR RNA evidenced by a rhodium probe. *Biochemistry* **34**: 6303-6309.
- Nigg, E. A. 1990. Mechanisms of signal transduction to the cell nucleus. *Cancer Research* **55**: 271-310.
- Nigg, E. A., Schäfer, G., Hilz, H. and Eppenberger, H. M. 1985a. Cyclic-AMP-dependent protein kinase type II is associated with the Golgi complex and with centrosomes. *Cell* **41**: 1039-1051.
- Nigg, E. A., Hilz, H., Eppenberger, H. M. and Dutly, F. 1985b. Rapid and reversible translocation of the catalytic subunit of cAMP-dependent protein kinase type II from the Golgi complex to the nucleus. *The EMBO Journal* **4**: 2801-2806.
- Nilsson, M. A. and Hultin, T. 1982. Analysis of the membrane-associated poly(A)⁺RNA in the cytoplasm of dormant *Artemia* cysts by DNA excess hybridization. *Biochimica et Biophysica Acta* **696**: 253-259.
- Noller, H. F. 1993. Peptidyl transferase: protein, ribonucleoprotein, or RNA? *Journal of Bacteriology* **175**: 5297-5300.

- Nomura, M. 1990. History of ribosome research: a personal account. In *The Ribosome: Structure, Function, & Evolution* (W. E. Hill, A. Dahlberg, R. A. Garrett, P. B. Moore, D. Schlessinger and J. R. Warner eds) pp 3-55. American Society for Microbiology, Washington, D. C.
- Nomura, M., Gourse, R. and Baughman, G. 1984. Regulation of the synthesis of ribosomes and ribosomal components. *Annual Review of Biochemistry* **53**: 75-117.
- Nygård, O. and Nilsson, L. 1987. The ribosomal binding site for elongation factor EF-2 contains 5 S ribosomal RNA. *Biochimica et Biophysica Acta* **908**: 46-53.
- Oakes, M. I., Scheinman, A., Atha, T., Shankweiler, G. and Lake, J. A. 1990. Ribosome structure: three-dimensional locations of rRNA and proteins. In *The Ribosome: Structure, Function & Evolution* (W. E. Hill, A. Dahlberg, R. A. Garrett, P. B. Moore, D. Schlessinger and J. R. Warner eds) pp 180-193. American Society for Microbiology, Washington, D. C.
- O'Brien, C. A. and Wolin, S. L. 1994. A possible role for the 60-kD Ro autoantigen in a discard pathway for defective 5S rRNA precursors. *Genes & Development* **8**: 2891-2903.
- Ochs, R. L., Lischwe, M. A., Spohn, W. H. and Busch, H. 1985. Fibrillarin: a new protein of the nucleolus identified by autoimmune sera. *Biology of the Cell* **54**: 123-134.
- Ochs, R. L., Stein, T. W. and Tan, E. M. 1994. Coiled bodies in the nucleolus of breast cancer cells. *Journal of Cell Science* **107**: 385-399.
- Ogata, K., Kurahashi, A., Tanaka, S., Ohsue, H. and Terao, K. 1991a. Occurrence of 5S rRNA in high molecular weight complexes of aminoacyl-tRNA synthetases in rat liver supernatant. *Journal of Biochemistry* **110**: 1030-1036.
- Ogata, K., Kurahashi, A., Kenmochi, N. and Terao, K. 1991b. Role of 5S rRNA as a positive effector of some aminoacyl-tRNA synthetases in macromolecular complexes, with specific reference to methionyl-tRNA synthetase. *Journal of Biochemistry* **110**: 1037-1044.
- Ogata, K., Kurahashi, A., Nishiyama, C. and Terao, K. 1993. Metabolic characteristics of rat liver cytosolic 5SRNA. *European Journal of Biochemistry* **213**: 1277-1282.
- Ogata, K., Kurahashi, A., Nishiyama, C. and Terao, K. 1994. Presence and role of the 5SrRNA-L5 protein complex (5SRNP) in the threonyl- and histidyl-tRNA synthetase complex in rat liver cytosol. *Biochimica et Biophysica Acta* **1218**: 388-400.

- Ogata, K., Kurahashi, A., Ohno, R., Takahashi, K. and Terao, K. 1995. Interaction of 5S rRNA-L5 protein complex, methionyl-tRNA, and methionyl-tRNA synthetase in the macromolecular ARS complex. *Journal of Biochemistry* **117**: 750-757.
- Olink-Coux, M., Arcangeletti, C., Pinardi, F., Minisini, R., Huesca, M., Chezzi, C. and Scherrer, K. 1994. Cytolocalization of prosome antigens on intermediate filament subnetworks of cytokeratin, vimentin and desmin type. *Journal of Cell Science* **107**: 353-366.
- Olson, M. O. J., Rivers, Z. M., Thompson, B. A., Kao, W.-Y. and Case, S. T. 1983. Interaction of nucleolar phosphoprotein C23 with cloned segments of rat ribosomal deoxyribonucleic acid. *Biochemistry* **22**: 3345-3351.
- O'Mahony, D. J., Xie, W.-Q., Smith, S. D., Singer, H. A. and Rothblum, L. I. 1992. Differential phosphorylation and localization of the transcription factor UBF *in vivo* in response to serum deprivation: *in vitro* dephosphorylation of UBF reduces its transactivation properties. *The Journal of Biological Chemistry* **267**: 35-38.
- Ornelles, D. A., Fey, E. G. and Penman, S. 1986. Cytochalasin releases mRNA from the cytoskeletal framework and inhibits protein synthesis. *Molecular and Cellular Biology* **6**: 1650-1662.
- Pace, B., Matthews, E. A., Johnson, K. D., Cantor, C. R. and Pace, N. R. 1982. Conserved 5S rRNA complement to tRNA is not required for protein synthesis. *Proceedings of the National Academy of Sciences USA* **79**: 36-40.
- Paine, P. L., Yassin, R., Paine, T. M., Vancurova, I., George, C. and Sahay, N. 1995. Intranuclear binding of nucleoplasmin. *Journal of Cellular Biochemistry* **58**: 105-114.
- Palecek, J., Habrova, V., Nedvidek, J. and Romanovsky, A. 1985. Dynamics of tubulin structures in *Xenopus laevis* oogenesis. *Journal of Embryology and Experimental Morphology* **87**: 75-86.
- Peattie, D. A., Douthwaite, S., Garrett, R. A. and Noller, H. F. 1981. A "bulged" double helix in a RNA-protein contact site. *Proceedings of the National Academy of Sciences USA* **78**: 7331-7335.
- Peculis, B. A. and Gall, J. G. 1992. Localization of the nucleolar protein NO38 in amphibian oocytes. *Journal of Cell Biology* **116**: 1-14.
- Peculis, B. A. and Steitz, J. A. 1993. Disruption of U8 nucleolar snRNA inhibits 5.8S and 28S rRNA processing in the *Xenopus* oocyte. *Cell* **73**: 1233-1245.

- Pelham, H. R. B. and Brown, D. D.** 1980. A specific transcription factor that can bind either the 5S RNA gene or 5S RNA. *Proceedings of the National Academy of Sciences USA* **77**: 4170-4174.
- Pelham, H. R. B., Wormington, W. M. and Brown, D. D.** 1981. Related 5S RNA transcription factors in *Xenopus* oocytes and somatic cells. *Proceedings of the National Academy of Sciences USA* **78**: 1760-1764.
- Peppel, K. and Baglioni, C.** 1990. A simple and fast method to extract RNA from tissue culture cells. *BioTechniques* **9**: 711-713.
- Perkowska, E., Macgregor, H. C. and Birnstiel, M. L.** 1968. Gene amplification in the oocyte nucleus of mutant and wild-type *Xenopus laevis*. *Nature* **217**: 649-650.
- Peters, R.** 1984. Nucleo-cytoplasmic flux and intracellular mobility in single hepatocytes measured by fluorescence microphotolysis. *The EMBO Journal* **3**: 1831-1836.
- Peterson, R. C., Doering, J. L. and Brown, D. D.** 1980. Characterization of two *Xenopus* somatic 5S DNAs and one minor oocyte-specific 5S DNA. *Cell* **20**: 131-141.
- Picard, B., le Maire, M., Wegnez, M. and Denis, H.** 1980. Biochemical research on oogenesis: composition of the 42-S storage particles of *Xenopus laevis* oocytes. *European Journal of Biochemistry* **109**: 359-368.
- Picard, B. and Wegnez, M.** 1979. Isolation of a 7S particle from *Xenopus laevis* oocytes: A 5S RNA-protein complex. *Proceedings of the National Academy of Sciences USA* **76**: 241-245.
- Pieler, T. and Erdmann, V. A.** 1982. Three-dimensional structural model of eubacterial 5S RNA that has functional implications. *Proceedings of the National Academy of Sciences USA* **79**: 4599-4603.
- Pieler, T. and Theunissen, O.** 1993. TFIIIA: nine fingers - three hands? *Trends in Biochemical Sciences* **18**: 226-229.
- Pokrywka, N. J. and Stephenson, E. C.** 1991. Microtubules mediate the localization of *bicoid* during *Drosophila* oogenesis. *Development* **113**: 55-66.
- Pokrywka, N. J. and Stephenson, E. C.** 1994. Localized RNAs are enriched in cytoskeletal extracts of *Drosophila* oocytes. *Developmental Biology* **166**: 210-219.
- Pokrywka, N. J. and Stephenson, E. C.** 1995. Microtubules are a general component of mRNA localization systems in *Drosophila* oocytes. *Developmental Biology* **167**: 363-370.

- Pondel, M. D. and King, M. L. 1988. Localized maternal mRNA related to transforming growth factor β mRNA is concentrated in a cytokeratin-enriched fraction from *Xenopus* oocytes. *Proceedings of the National Academy of Sciences USA* **85**: 7612-7616.
- Pratt, W. B. 1992. Control of steroid receptor function and cytoplasmic nuclear transport by heat shock proteins. *BioEssays* **14**: 841-848.
- Pruijn, G. J. M., Slobbe, R. L. and van Venrooij, W. J. 1991. Analysis of protein-RNA interactions within Ro ribonucleoprotein complexes. *Nucleic Acids Research* **19**: 5173-5180.
- Radu, A., Blobel, G. and Moore, M. S. 1995. Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins. *Proceedings of the National Academy of Sciences USA* **92**: 1769-1773.
- Ranjan, M., Tafuri, S. R. and Wolffe, A. P. 1993. Masking mRNA from translation in somatic cells. *Genes & Development* **7**: 1725-1736.
- Raška, I., Andrade, L. E. C., Ochs, R. L., Chan, E. K. L., Chang, C.-M., Roos, G. and Tan, E. M. 1991. Immunological and ultrastructural studies of the nuclear coiled body with autoimmune antibodies. *Experimental Cell Research* **195**: 27-37.
- Raška, I., Dundr, M., Koberna, K., Melcák, I., Risueño, M.-C. and Török, I. 1995. Does the synthesis of ribosomal RNA take place within nucleolar fibrillar centers or dense fibrillar components? A critical appraisal. *Journal of Structural Biology* **114**: 1-22.
- Rebagliati, M. R., Weeks, D. L., Harvey, R. P. and Melton, D. A. 1985. Identification and cloning of localized maternal RNAs from *Xenopus* eggs. *Cell* **42**: 769-777.
- Reddy, B. A., Kloc, M. and Etkin, L. 1991. The cloning and characterization of a maternally expressed novel zinc finger nuclear phosphoprotein (xnf7) in *Xenopus laevis*. *Developmental Biology* **148**: 107-116.
- Reddy, B. A., Kloc, M. and Etkin, L. D. 1992. The cloning and characterization of a localized maternal transcript in *Xenopus laevis* whose zygotic counterpart is detected in the CNS. *Mechanisms of Development* **39**: 143-150.
- Redmond, T., Sanchez, E. R., Bresnick, E. H., Schlesinger, M. J., Toft, D. O. and Pratt, W. B. 1989. Immunofluorescence colocalization of the 90 kDa heat-shock protein and microtubules in interphase and mitotic mammalian cells. *European Journal of Cell Biology* **50**: 66-75.

- Reimer, G., Raška, I., Scheer, U. and Tan, E. M.** 1988. Immunolocalization of 7-2-ribonucleoprotein in the granular component of the nucleolus. *Experimental Cell Research* **176**: 117-128.
- Rhim, H. and Rice, A. P.** 1994. Functional significance of the dinucleotide bulge in stem-loop 1 and stem-loop 2 of HIV-2 TAR RNA. *Virology* **202**: 202-211.
- Rihs, H.-P., Jans, D. A., Fan, H. and Peters, R.** 1991. The rate of nuclear cytoplasmic protein transport is determined by the casein kinase II site flanking the nuclear localization sequence of the SV40 T-antigen. *The EMBO Journal* **10**: 633-639.
- Rihs, H.-P. and Peters, R.** 1989. Nuclear transport kinetics depend on phosphorylation-site-containing sequences flanking the karyophilic signal of the Simian virus 40 T-antigen. *The EMBO Journal* **8**: 1479-1484.
- Risueño, M. C. and Testillano, P. S.** 1994. Cytochemistry and immunocytochemistry of nucleolar chromatin in plants. *Micron* **25**: 331-360.
- Robbins, J., Dilworth, S. M., Laskey, R. A. and Dingwall, C.** 1991. Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell* **64**: 615-623.
- Rogers, M. E.** 1968. Ribonucleoprotein particles in the amphibian oocyte nucleus. *Journal of Cell Biology* **36**: 421-432.
- Romaniuk, P. J.** 1985. Characterization of the RNA binding properties of transcription factor IIIA of *Xenopus laevis* oocytes. *Nucleic Acids Research* **13**: 5369-5387.
- Romaniuk, P. J.** 1989. The role of highly conserved single-stranded nucleotides of *Xenopus* 5S RNA in the binding of transcription factor IIIA. *Biochemistry* **28**: 1388-1395.
- Romaniuk, P. J., Leal de Stevenson, I., Ehresmann, C., Romby, P. and Ehresmann, B.** 1988. A comparison of the solution structures and conformational properties of the somatic and oocyte 5S rRNAs of *Xenopus laevis*. *Nucleic Acids Research* **16**: 2295-2312.
- Romaniuk, P. J., Leal de Stevenson, I. and Wong, H.-H. A.** 1987a. Defining the binding site of *Xenopus* transcription factor IIIA on 5S RNA using truncated and chimeric molecules. *Nucleic Acids Research* **15**: 2737-2755.
- Romaniuk, P. J., Lowary, P., Wu, H.-N., Stormo, G. and Uhlenbeck, O. C.** 1987b. RNA binding site of R17 coat protein. *Biochemistry* **26**: 1563-1568.

- Romby, P., Baudin, F., Brunel, C., Leal de Stevenson, I., Westhof, E., Romaniuk, P. J., Ehresmann, C. and Ehresmann, B. 1990. Ribosomal 5S RNA from *Xenopus laevis* oocytes: conformation and interaction with transcription factor IIIA. *Biochimie* **72**: 437-452.
- Roth, S., Hiromi, Y., Godt, D. and Nüsslein-Volhard, C. 1991. *cactus*, a maternal gene required for proper formation of the dorsoventral morphogen gradient in *Drosophila* embryos. *Development* **112**: 371-388.
- Ryabova, L. V., Virtanen, L., Olink-Coux, M., Scherrer, K. and Vassetzky, S. G. 1994. Distribution of prosome proteins and their relationship with the cytoskeleton in oogenesis of *Xenopus laevis*. *Molecular Reproduction and Development* **37**: 195-203.
- St Johnston, D. 1995. The intracellular localization of messenger RNAs. *Cell* **81**: 161-170.
- Saitoh, H. and Dasso, M. 1995. The RCC1 protein interacts with Ran, RanBP1, hsc70, and a 340-kDa protein in *Xenopus* extracts. *The Journal of Biological Chemistry* **270**: 10658-10663.
- Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory Press, New York.
- Sarge, K. and Maxwell, E. S. 1991. Intermolecular hybridization of 5S rRNA with 18S rRNA: Identification of a 5'-terminally-located nucleotide sequence in mouse 5S rRNA which base-pairs with two specific complementary sequences in 18S rRNA. *Biochimica et Biophysica Acta* **1088**: 57-70.
- Savino, R. and Gerbi, S. A. 1990. *In vivo* disruption of *Xenopus* U3 snRNA affects ribosomal RNA processing. *The EMBO Journal* **9**: 2299-2308.
- Savino, R., Hitti, Y. and Gerbi, S. A. 1992. Genes for *Xenopus laevis* U3 small nuclear RNA. *Nucleic Acids Research* **20**: 5435-5442.
- Scheer, U. and Benevente, R. 1990. Functional and dynamic aspects of the mammalian nucleolus. *BioEssays* **12**: 14-21.
- Scherrer, K. 1990. Prosomes, subcomplexes of untranslated mRNP. *Molecular Biology Reports* **14**: 1-9.
- Schimmang, T., Tollervey, D., Kern, H., Frank, R. and Hurt, E. C. 1989. A yeast nucleolar protein related to mammalian fibrillarin is associated with small nucleolar RNA and is essential for viability. *The EMBO Journal* **8**: 4015-4024.
- Schliwa, M. 1986. *The cytoskeleton: An Introductory Survey*. Springer-Verlag, Vienna.

- Schmidt, C., Lipsius, E. and Kruppa, J. 1995. Nuclear and nucleolar targeting of human ribosomal protein S6. *Molecular Biology of the Cell* **6**: 1875-1885.
- Schmidt-Zachmann, M. S., Dargemont, C., Kühn, L. C. and Nigg, E. A. 1993. Nuclear export of proteins: The role of nuclear retention. *Cell* **74**: 493-504.
- Schmidt-Zachmann, M. S., Hügle, B., Scheer, U. and Franke, W. W. 1984. Identification and localization of a novel nucleolar protein of high molecular weight by a monoclonal antibody. *Experimental Cell Research* **153**: 327-346.
- Schmidt-Zachmann, M. S., Hügle-Dörr, B. and Franke, W. W. 1987. A constitutive nucleolar protein identified as a member of the nucleoplasmin family. *The EMBO Journal* **6**: 1881-1890.
- Schmidt-Zachmann, M. S. and Nigg, E. A. 1993. Protein localization to the nucleolus: a search for targeting domains in nucleolin. *Journal of Cell Science* **105**: 799-806.
- Schmitz, M. L., Henkel, T. and Baeuerle, P. A. 1991. Proteins controlling the nuclear uptake of NF- κ B, Rel and dorsal. *Trends in Cell Biology* **1**: 130-137.
- Schöfer, C., Müller, M., Leitner, M. D. and Wachtler, F. 1993. The uptake of uridine in the nucleolus occurs in the dense fibrillar component: immunogold localization of incorporated digoxigenin-UTP at the electron microscopic level. *Cytogenetics and Cell Genetics* **64**: 27-30.
- Schumacher, J. M., Lee, K., Edelhoff, S. and Braun, R. E. 1995. Spnr, a murine RNA-binding protein that is localized to cytoplasmic microtubules. *Journal of Cell Biology* **129**: 1023-1032.
- Schwartz, S. P., Aisenthal, L., Elisha, Z., Oberman, F. and Yisraeli, J. K. 1992. A 69-kDa RNA-binding protein from *Xenopus* oocytes recognizes a common motif in two vegetally localized maternal RNAs. *Proceedings of the National Academy of Sciences USA* **89**: 11895-11899.
- Scripture, J. B. and Huber, P. W. 1995. Analysis of the binding of *Xenopus* ribosomal protein L5 to oocyte 5 S rRNA. *The Journal of Biological Chemistry* **270**: 27358-27365.
- Shan, X., Xue, Z. and Mélése, T. 1994. YeastNPI46 encodes a novel prolyl *cis-trans* isomerase that is located in the nucleolus. *Journal of Cell Biology* **126**: 853-862.
- Sharpless, K., Biegel, D., Yang, T. and Pachter, J. S. 1993. β -actin mRNA-binding proteins associated with the cytoskeletal framework. *European Journal of Biochemistry* **212**: 217-225.

- Shastry, B. S., Honda, B. M. and Roeder, R. G. 1984. Altered levels of a 5 S gene-specific transcription factor (TFIIIA) during oogenesis and embryonic development of *Xenopus laevis*. *The Journal of Biological Chemistry* **259**: 11373-11382.
- Shatsky, I. N., Evstafieva, A. G., Bystrova, T. F., Bogdanov, A. A. and Vasiliev, V. D. 1980. Topography of RNA in the ribosome: location of the 3'-end of 5 S RNA on the central protuberance of the 50S subunit. *FEBS Letters* **121**: 97-100.
- Shaw, P. J., Highett, M. I., Beven, A. F. and Jordan, E. G. 1995. The nucleolar architecture of polymerase I transcription and processing. *The EMBO Journal* **14**: 2896-2906.
- Shiokawa, K. 1983. Mobilization of maternal mRNA in amphibian eggs with special reference to the possible role of membraneous supramolecular structures. *FEBS Letters* **151**: 179-184.
- Simons, J., De Herdt, E., Kondo, M. and Slegers, H. 1978. Membrane associated poly(A)-containing ribonucleoproteins in *Artemia salina* gastrula embryos. *FEBS Letters* **91**: 53-56.
- Simos, G. and Hurt, E. C. 1995. Nucleocytoplasmic transport: factors and mechanisms. *FEBS Letters* **369**: 107-112.
- Singer, R. H., Langevin, G. L. and Lawrence, J. B. 1989. Ultrastructural visualization of cytoskeletal mRNAs and their associated proteins using double-label in situ hybridization. *Journal of Cell Biology* **108**: 2343-2353.
- Siomi, H., Shida, H., Maki, M. and Hatanaka, M. 1990. Effects of a highly basic region of human immunodeficiency virus Tat protein on nucleolar localization. *Journal of Virology* **64**: 1803-1907.
- Siomi, H., Shida, H., Nam, S. H., Nosaka, T., Maki, M. and Hatanaka, M. 1988. Sequence requirements for nucleolar localization of human T cell leukemia virus type I pX protein, which regulates viral RNA processing. *Cell* **55**: 197-209.
- Specht, T., Wolters, J. and Erdmann, V. A. 1990. Compilation of 5S rRNA and 5S rRNA gene sequences. *Nucleic Acids Research* **18**, suppl.: 2215-2230.
- Spector, D. L., Ochs, R. L. and Busch, H. 1984. Silver staining, immunofluorescence, and immunoelectron microscopic localization of nucleolar phosphoproteins B23 and C23. *Chromosoma* **90**: 139-148.
- Steitz, J. A., Berg, C., Hendrick, J. P., La Branche-Chabot, H., Metspalu, A., Rinke, J. and Yario, T. 1988. A 5S rRNA/L5 complex is a precursor to ribosome assembly in mammalian cells. *Journal of Cell Biology* **106**: 545-556.

- Steitz, T. A. 1993. Similarities and differences between RNA and DNA recognition by proteins. In *The RNA World* (R. F. Gesteland and J. F. Atkins eds) pp 219-237. Cold Spring Harbor Laboratory Press, New York.
- Stochaj, U. and Silver, P. 1992. Nucleocytoplasmic traffic of proteins. *European Journal of Cell Biology* **59**: 1-11.
- Stöffler-Meilicke, M. and Stöffler, G. 1990. Topography of the ribosomal proteins from *Escherichia coli* within the intact subunits as determined by immunoelectron microscopy and protein-protein cross-linking. In *The Ribosome: Structure, Function & Evolution* (W. E. Hill, A. Dahlberg, R. A. Garrett, P. B. Moore, D. Schlessinger and J. R. Warner eds) pp 123-133. American Society for Microbiology, Washington, D. C.
- Stöffler-Meilicke, M., Stöffler, G., Odom, O. W., Zinn, A., Kramer, G. and Hardesty, B. 1981. Localization of 3' ends of 5S and 23S rRNAs in reconstituted subunits of *Escherichia coli* ribosomes. *Proceedings of the National Academy of Sciences USA* **78**: 5538-5542.
- Sun, C. and Woolford, J. L. J. 1994. The yeast *NOP4* gene product is an essential nucleolar protein required for pre-rRNA processing and accumulation of 60S ribosomal subunits. *The EMBO Journal* **13**: 3127-3135.
- Sundell, C. L. and Singer, R. H. 1990. Actin mRNA localizes in the absence of protein synthesis. *Journal of Cell Biology* **111**: 2397-2403.
- Suprenant, K. A. 1993. Microtubules, ribosomes, and RNA: Evidence for cytoplasmic localization and translational regulation. *Cell Motility and the Cytoskeleton* **25**: 1-9.
- Szebeni, A., Herrera, J. E. and Olson, M. O. J. 1995. Interaction of nucleolar protein B23 with peptides related to nuclear localization signals. *Biochemistry* **34**: 8037-8042.
- Szewczak, A. A., Moore, P. B., Chan, Y.-L. and Wool, I. G. 1993. The conformation of the sarcin/ricin loop from 28S ribosomal RNA. *Proceedings of the National Academy of Sciences USA* **90**: 9581-9585.
- Tafuri, S. R. and Wolffe, A. P. 1993. Selective recruitment of masked maternal mRNA from messenger ribonucleoprotein particles containing FRGY2 (mRNP4). *The Journal of Biological Chemistry* **268**: 24255-24261.
- Takemura, M., Ohta, N., Furuichi, Y., Takahashi, T., Yoshida, S., Olson, M. O. J. and Umekawa, H. 1994. Stimulation of calf thymus DNA polymerase α activity by nucleolar protein B23. *Biochemical and Biophysical Research Communications* **199**: 46-51.

- Taneja, K. L., Lifshitz, L. M., Fay, F. S. and Singer, R. H. 1992. Poly(A) RNA codistribution with microfilaments: Evaluation by in situ hybridization and quantitative digital imaging microscopy. *Journal of Cell Biology* **119**: 1245-1260.
- Terns, M. P. and Dahlberg, J. E. 1994. Retention and 5' cap trimethylation of U3 snRNA in the nucleus. *Science* **264**: 959-961.
- Terns, M. P., Grimm, C., Lund, E. and Dahlberg, J. E. 1995. A common maturation pathway for small nucleolar RNAs. *The EMBO Journal* **14**: 4860-4871.
- Theunissen, O., Rudt, F., Guddat, U., Mentzel, H. and Pieler, T. 1992. RNA and DNA binding zinc fingers in *Xenopus* TFIIIA. *Cell* **71**: 679-690.
- Theurkauf, W. E., Alberts, B. M., Jan, Y. N. and Jongens, T. A. 1993. A central role for microtubules in the differentiation of *Drosophila* oocytes. *Development* **118**: 1169-1180.
- Thomas, C. 1972. Correlation between ultrastructural aspect of nucleoli and inhibition of ribosomal RNA synthesis in *Xenopus laevis* oocytes. *Experimental Cell Research* **74**: 547-551.
- Thomsen, G. H. and Melton, D. A. 1993. Processed Vg1 protein is an axial mesoderm inducer in *Xenopus*. *Cell* **74**: 433-441.
- Tollervey, D., Lehtonen, H., Jansen, R., Kern, H. and Hurt, E. C. 1993. Temperature-sensitive mutations demonstrate roles for yeast fibrillarin in pre-rRNA processing, pre-rRNA methylation, and ribosome assembly. *Cell* **72**: 443-457.
- Tuma, R. S., Stolk, J. A. and Roth, M. B. 1993. Identification and characterization of a sphere organelle protein. *Journal of Cell Biology* **122**: 767-773.
- Tyc, K. and Steitz, J. A. 1989. U3, U8 and U13 comprise a new class of mammalian snRNPs localized in the cell nucleolus. *The EMBO Journal* **8**: 3113-3119.
- Tycowski, K. T., Shu, M.-D. and Steitz, J. A. 1994. Requirement for intron-encoded U22 small nucleolar RNA in 18S ribosomal RNA maturation. *Science* **266**: 1558-1561.
- Uchiumi, T., Kikuchi, M. and Ogata, K. 1986. Cross-linking study on protein neighborhoods at the subunit interface of rat liver ribosomes with 2-iminothiolane. *The Journal of Biological Chemistry* **261**: 9663-9667.
- Valdez, B. C., Perlaky, L., Henning, D., Saijo, Y., Chan, P.-K. and Busch, H. 1994. Identification of the nuclear and nucleolar localization signals of the protein p120: interaction with translocation protein B23. *The Journal of Biological Chemistry* **269**: 23776-23783.

- Vancurova, I., Lou, W., Paine, T. M. and Paine, P. L. 1993. Nucleoplasmin uptake by facilitated transport and intranuclear binding. *European Journal of Cell Biology* **62**: 22-33.
- Van Gansen, P. and Schram, A. 1972. Evolution of the nucleoli during oogenesis in *Xenopus laevis* studied by electron microscopy. *Journal of Cell Science* **10**: 339-367.
- Vankan, P., McGuigan, C. and Mattaj, I. W. 1990. Domains of U4 and U6 snRNAs required for snRNP assembly and splicing complementation in *Xenopus* oocytes. *The EMBO Journal* **9**: 3397-3404.
- Van Ryk, D. I., Lee, Y. and Nazar, R. N. 1992. Unbalanced ribosome assembly in *Saccharomyces cerevisiae* expressing mutant 5 S RNAs. *The Journal of Biological Chemistry* **267**: 16177-16181.
- Vedeler, A., Pryme, I. F. and Hesketh, J. E. 1991. The characterization of free, cytoskeletal and membrane-bound polysomes in Krebs II ascites and 3T3 cells. *Molecular and Cellular Biochemistry* **100**: 183-193.
- Viel, A., Armand, M.-J., Callen, J. C., De Gracia, A. G., Denis, H. and le Maire, M. 1990. Elongation factor 1 α (EF-1 α) is concentrated in the balbiani body and accumulates coordinately with the ribosomes during oogenesis of *Xenopus laevis*. *Developmental Biology* **141**: 270-278.
- Wachtler, F. and Stahl, A. 1993. The nucleolus: a structural and functional interpretation. *Micron* **24**: 473-505.
- Wallace, R. A., Jared, D. W., Dumont, J. N. and Sega, M. W. 1973. Protein incorporation by isolated amphibian oocytes: optimum incubation conditions. *Experimental Zoology* **184**: 321-334.
- Walleczek, J., Schüler, D., Stöffler-Meilicke, M., Brimacombe, R. and Stöffler, G. 1988. A model for the spatial arrangement of the proteins in the large subunit of the *Escherichia coli* ribosome. *The EMBO Journal* **7**: 3571-3576.
- Walter, P. and Blobel, G. 1981a. Translocation of proteins across the endoplasmic reticulum II. Signal recognition protein (SRP) mediates the selective binding to microsomal membranes of in-vitro assembled polysomes synthesising secretory protein. *Journal of Cell Biology* **91**: 551-556.
- Walter, P. and Blobel, G. 1981b. Translocation across the endoplasmic reticulum III. Signal recognition protein (SRP) causes signal-sequence dependent and site-specific arrest of chain elongation that is released by microsomal membranes. *Journal of Cell Biology* **91**: 557-561.

- Walter, P., Ibrahimi, I. and Blobel, G. 1981. Translocation of proteins across the endoplasmic reticulum I. Signal recognition protein (SRP) binds to in-vitro assembled polysomes synthesising secretory protein. *Journal of Cell Biology* **91**: 545-550.
- Walter, P. and Johnson, A. E. 1994. Signal sequence recognition and protein targeting to the endoplasmic reticulum membrane. *Annual Review of Cell Biology* **10**: 87-119.
- Wang, C. and Lehmann, R. 1991. Nanos is the localized posterior determinant in *Drosophila*. *Cell* **66**: 637-647.
- Wang, D., Baumann, A., Szebeni, A. and Olson, M. O. J. 1994. The nucleic acid binding activity of nucleolar protein B23.1 resides in its carboxy-terminal end. *The Journal of Biological Chemistry* **269**: 30994-30998.
- Warner, J. R. 1990. The nucleolus and ribosome formation. *Current Opinion in Cell Biology* **2**: 521-527.
- Warner, J. R. and Soeiro, R. 1967. Nascent ribosomes from HeLa cells. *Proceedings of the National Academy of Sciences USA* **58**: 1984-1990.
- Weeks, D. L. and Melton, D. A. 1987a. A maternal mRNA localized to the vegetal hemisphere in *Xenopus* eggs codes for a growth factor related to TGF- β . *Cell* **51**: 861-867.
- Weeks, D. L. and Melton, D. A. 1987b. A maternal mRNA localized to the animal pole of *Xenopus* eggs encodes a subunit of mitochondrial ATPase. *Proceedings of the National Academy of Sciences USA* **84**: 2798-2802.
- Weeks, K. M. and Crothers, D. M. 1991. RNA recognition by Tat-derived peptides: Interaction in the major groove? *Cell* **66**: 577-588.
- Weeks, K. M. and Crothers, D. M. 1993. Major groove accessibility of RNA. *Science* **261**: 1574-1577.
- Westhof, E., Romby, P., Romaniuk, P. J., Ebel, J.-P., Ehresmann, C. and Ehresmann, B. 1989. Computer modelling from solution data of spinach chloroplast and of *Xenopus laevis* somatic and oocyte 5S rRNAs. *Journal of Molecular Biology* **207**: 417-431.
- Wikström, A.-C., Bakke, O., Okret, S., Brönnegård, M. and Gustaffson, J.-A. 1987. Intracellular localization of the glucocorticoid receptor: evidence for cytoplasmic and nuclear localization. *Endocrinology* **120**: 1232-1242.
- Wimberly, B. 1994. A common RNA loop motif as a docking module and its function in the hammerhead ribozyme. *Nature Structural Biology* **1**: 820-827.

- Wimberly, B., Varani, G. and Tinoco, I. J. 1993. The conformation of loop E of eukaryotic 5S ribosomal RNA. *Biochemistry* **32**: 1078-1087.
- Wolffe, A. P. 1994. The role of transcription factors, chromatin structure and DNA replication in 5 S RNA gene regulation. *Journal of Cell Science* **107**: 2055-2063.
- Wolffe, A. P. and Brown, D. D. 1988. Developmental regulation of two 5S ribosomal RNA genes. *Science* **241**: 1626-1631.
- Wormington, W. M. 1989. Developmental expression and 5S rRNA-binding activity of *Xenopus laevis* ribosomal protein L5. *Molecular and Cellular Biology* **9**: 5281-5288.
- Wormington, W. M. and Baum, E. Z. 1986. Developmental regulation of *Xenopus* ribosomal protein genes. In *Gametogenesis and the Early Embryo* pp 347-356. Alan R. Liss, Inc., New York.
- Wu, C.-H. H. and Gall, J. G. 1993. U7 small nuclear RNA in C snurposomes of the *Xenopus* germinal vesicle. *Proceedings of the National Academy of Sciences USA* **90**: 6257-6259.
- Wu, H., Jiang, L. and Zimmermann, R. A. 1994. The binding site for ribosomal protein S8 in 16S rRNA and *spc* mRNA from *Escherichia coli*: minimum structural requirements and the effects of single bulged bases on S8-RNA interaction. *Nucleic Acids Research* **22**: 1687-1695.
- Wu, H.-N. and Uhlenbeck, O. C. 1987. Role of a bulged A residue in a specific RNA-protein interaction. *Biochemistry* **26**: 8221-8227.
- Wu, Z., Murphy, C., Callan, H. G. and Gall, J. G. 1991. Small nuclear ribonucleoproteins and heterogeneous nuclear ribonucleoproteins in the amphibian germinal vesicle: loops, spheres, and snurposomes. *Journal of Cell Biology* **113**: 465-483.
- Wyatt, J. R., Puglisi, J. D. and Tinoco, I., Jr. 1989. RNA folding: Pseudoknots, loops and bulges. *BioEssays* **11**: 100-106.
- Wyatt, J. R. and Tinoco, I., Jr. 1993. RNA structural elements and RNA function. In *The RNA World* (F. Gesteland and J. F. Atkins eds) pp 465-496. Cold Spring Harbor Laboratory Press, New York.
- Xue, Z., Shan, X., Lapeyre, B. and Mèlèse, T. 1993. The amino terminus of mammalian nucleolin specifically recognizes SV40 T-antigen type nuclear localization sequences. *European Journal of Cell Biology* **62**: 13-21.

- Yaguchi, M., Rollin, C. F., Roy, C. and Nazar, R. N.** 1984. The 5S RNA binding protein from yeast (*Saccharomyces cerevisiae*) ribosomes: an RNA binding sequence in the carboxy-terminal region. *European Journal of Biochemistry* **139**: 451-457.
- Yan, C. and Mélése, T.** 1993. Multiple regions of NSR1 are sufficient for accumulation of a fusion protein within the nucleolus. *Journal of Cell Biology* **123**: 1081-1091.
- Yeh, L.-C. C. and Lee, J. C.** 1995a. An *in vitro* system for studying RNA-protein interaction: application to a study of yeast ribosomal protein L1 binding to 5S rRNA. *Biochimie* **77**: 167-173.
- Yeh, L.-C. C. and Lee, J. C.** 1995b. Contributions of multiple basic amino acids in the C-terminal region of yeast ribosomal protein L1 to 5 S rRNA binding and 60 S ribosome stability. *Journal of Molecular Biology* **246**: 295-307.
- Yisraeli, J. K. and Melton, D. A.** 1988. The maternal mRNA Vg1 is correctly localized following injection into *Xenopus* oocytes. *Nature* **336**: 592-595.
- Yisraeli, J. K., Sokol, S. and Melton, D. A.** 1990. A two-step model for the localization of maternal mRNA in *Xenopus* oocytes: Involvement of microtubules and microfilaments in the translocation and anchoring of Vg1 mRNA. *Development* **108**: 289-298.
- You, Q. and Romaniuk, P. J.** 1990. The effects of disrupting 5S RNA helical structures on the binding of *Xenopus* transcription factor IIIA. *Nucleic Acids Research* **18**: 5055-5062.
- Yu, R. S. T. and Wittmann, H. G.** 1973. The sequence of steps in the attachment of 5-S RNA to cores of *Escherichia coli* ribosomes. *Biochimica et Biophysica Acta* **324**: 375-385.
- Yung, B. Y.-M., Busch, H. and Chan, P.-K.** 1985. Translocation of nucleolar phosphoprotein B23 (37 kDa / pI 5.1) induced by selective inhibitors of ribosome synthesis. *Biochimica et Biophysica Acta* **826**: 167-173.
- Zabel, U., Henkel, T., dos Santos Silva, M. and Baeuerle, P. A.** 1993. Nuclear uptake control of NF- κ B by MAD-3, an I κ B protein present in the nucleus. *The EMBO Journal* **12**: 201-211.
- Zagorska, L., Van Duin, J., Noller, H. F., Pace, B., Johnson, K. D. and Pace, N. R.** 1984. The conserved 5 S rRNA complement to tRNA is not required for translation of natural mRNA. *The Journal of Biological Chemistry* **259**: 2798-2802.
- Zehavi-Willner, T. and Danon, D.** 1972. The isolation and properties of reticulocyte soluble 5 S RNA. *FEBS Letters* **26**: 151-156.

Zumbe, A., Stähli, C. and Trachsel, H. 1982. Association of a Mr 50,000 cap-binding protein with the cytoskeleton in baby hamster kidney cells. *Proceedings of the National Academy of Sciences USA* **79**: 2927-2931.

APPENDICES

^a The numbers in the designations refer to those 5S RNA nucleotides which have been substituted or deleted (Fig. 3-3).

^b Ratio of the apparent association constant (K_a) measured for the mutant 5S RNA divided by the K_a measured for wild-type 5S RNA. Data summarised from Baudin and Romaniuk, 1989; Romaniuk, 1989; You and Romaniuk, 1990; Baudin *et al.*, 1991.

^c Ratio of concentrations of mutant 5S RNA to wild-type 5S RNA required to give a 50% competition value. ND, not determined. Data summarised from Baudin and Romaniuk, 1989; Romaniuk, 1989; Baudin *et al.*, 1991.

^d Binding affinity of mutant 5S RNA made relative to wild-type. Data from Q. You, W. Q. Zang, P. J. Romaniuk, in prep.

^e Nuclear transport of mutant 5S RNA relative to wild-type after microinjection into the oocyte cytoplasm. Data from Allison *et al.*, 1993; L. A. Allison and P. J. Romaniuk, in prep.

APPENDIX I

SUMMARY OF MUTANT 5S RNA PHENOTYPES

Region of Molecule	5S RNA Mutant ^a	TFIIIA binding ^b	TFIIIA competition ^c	L5 binding ^d	Nuclear transport ^e
	Oocyte type	1.00	1.00	1.00	1.00
<u>Helix II</u>	14-15	0.85 ± 0.22	ND	1.11 ± 0.05	0.49 ± 0.03
	64-65	0.74 ± 0.24	ND	1.02 ± 0.13	0.97
	14-15/64-65	1.11 ± 0.32	ND	1.00 ± 0.28	1.04 ± 0.16
	16-21	0.32 ± 0.15	ND	0.82 ± 0.05	0.25 ± 0.05
	57-62	0.40 ± 0.15	ND	0.99 ± 0.03	0.73
	16-21/57-62	1.09 ± 0.48	ND	0.94 ± 0.13	1.88
<u>Helix III</u>	27-32	0.75 ± 0.10	ND	0.52 ± 0.15	0.70 ± 0.23
	45-52	0.76 ± 0.12	ND	0.54 ± 0.12	1.45 ± 0.34
<u>Helix IV</u>	78-81	0.88 ± 0.01	ND	1.11 ± 0.13	0.99
	95-98	0.78 ± 0.02	ND	0.99 ± 0.10	0.86
	78-81/95-98	0.86 ± 0.01	ND	1.00 ± 0.26	1.19 ± 0.46
	82-86	0.81 ± 0.30	ND	1.28 ± 0.19	1.75 ± 0.02
	91-94	0.96 ± 0.18	ND	1.06 ± 0.09	0.85 ± 0.31
	82-86/91-94	1.21 ± 0.35	ND	1.42 ± 0.26	1.38 ± 0.19
<u>Helix V</u>	67-70	0.75 ± 0.12	ND	1.18 ± 0.11	1.18 ± 0.44
	105-108	0.39 ± 0.06	ND	1.11 ± 0.01	0.46 ± 0.15
	67-70/105-108	0.71 ± 0.01	ND	1.03 ± 0.07	1.07 ± 0.34
	71-72	0.35 ± 0.21	ND	0.88 ± 0.17	0.44 ± 0.12
	103-104	0.50 ± 0.23	ND	1.08 ± 0.08	0.48 ± 0.18
	71-72/103-104	1.18 ± 0.32	ND	1.08 ± 0.10	1.37 ± 0.43
<u>Loop A</u>	10-13	0.30 ± 0.01	0.06 ± 0.01	1.19 ± 0.23	0.63 ± 0.00
<u>Loop B</u>	22-26	1.00 ± 0.02	1.24 ± 0.04	0.86 ± 0.05	1.18
<u>Loop C</u>	33-39	1.00 ± 0.02	1.75 ± 0.13	0.92 ± 0.41	0.97 ± 0.25
	41-44	0.40 ± 0.10	0.52 ± 0.05	1.03 ± 0.22	0.33 ± 0.06
<u>Loop D</u>	87-90	0.71 ± 0.10	1.45 ± 0.05	1.20 ± 0.02	1.26
<u>Loop E</u>	73-76	0.57 ± 0.02	0.38 ± 0.05	0.77 ± 0.25	1.60 ± 0.20
	96-101	0.59 ± 0.01	0.52 ± 0.05	0.63 ± 0.04	0.36 ± 0.04
<u>Bulged nucleotides</u>	Δ49,50	1.00 ± 0.02	1.00 ± 0.13	0.91 ± 0.02	0.76 ± 0.38
	Δ63	1.00 ± 0.02	1.00 ± 0.02	1.05 ± 0.18	1.04 ± 0.40
	Δ83	1.00 ± 0.05	1.00 ± 0.02	1.22 ± 0.25	0.88 ± 0.09
<u>Hinge nucleotides</u>	C66	0.12 ± 0.01	0.19 ± 0.02	ND	0.43 ± 0.05
	G109	0.17 ± 0.05	0.14 ± 0.10	ND	0.77 ± 0.02

APPENDIX II

NUCLEOLAR LOCALISATION OF 5S RNA MUTANTS

5S RNA Mutant	% Nucleolar ^a	Oocyte-type % No ^b
Oocyte-type	33.2 ± 1.4 (70)	
Somatic-type	70.1 ± 2.3 (14)	32.3 ± 1.8
<u>Helix II</u>		
14-15	25.1 ± 1.9 (14)	33.3 ± 2.4
64-65	27.9 ± 1.4 (11)	32.9 ± 2.8
16-21	21.7 ± 1.7 (11)	36.0 ± 2.3
57-62	50.9 ± 2.6 (6)	30.7 ± 2.5
16-21/57-62	31.0 ± 2.7 (6)	30.7 ± 2.5
<u>Helix III</u>		
27-32	21.5 ± 1.9 (7)	16.5 ± 0.7
45-52	38.6 ± 2.0 (8)	16.5 ± 0.7
<u>Helix IV</u>		
95-98	42.3 ± 2.6 (6)	21.5 ± 2.7
<u>Helix V</u>		
105-108	68.0 ± 2.7 (9)	52.3 ± 5.0
71-72	34.0 ± 3.2 (11)	32.9 ± 2.8
103-104	29.6 ± 2.5 (11)	25.8 ± 3.9
<u>Loop A</u>		
10-13	30.5 ± 3.9 (12)	34.4 ± 2.4
<u>Loop B</u>		
22-26	31.9 ± 2.5 (10)	18.9 ± 1.7
<u>Loop C</u>		
41-44	28.6 ± 3.3 (10)	35.0 ± 1.9
<u>Loop E</u>		
96-101	28.0 ± 2.7 (16)	44.5 ± 3.5
<u>Bulged Nucleotides</u>		
D49,50	78.6 ± 2.2 (14)	31.7 ± 1.8
D63	61.2 ± 3.0 (11)	46.6 ± 5.7
D83	36.7 ± 3.8 (11)	31.4 ± 2.6
<u>Hinge Nucleotides</u>		
C66	32.7 ± 2.4 (9)	30.5 ± 1.7
G109	31.2 ± 3.2 (9)	32.3 ± 1.5

^a Nucleolar localisation was calculated as a percentage of the RNA in the nucleus after injection into the oocyte cytoplasm. The number of replicates is shown in parentheses.

^b The percentage of nucleolar localisation (% No) for oocyte-type 5S RNA in the same batches of oocytes.

APPENDIX III

JOURNAL PUBLICATIONS

I. The attached reprint from *Molecular and Cellular Biology* contains part of the results presented in this thesis. My contribution to this publication consisted of immunoprecipitation and non-denaturing gel electrophoresis assays, to determine the ability of a series of mutant 5S RNA molecules to be incorporated into oocyte ribonucleoprotein particles. These data were presented in Chapter 3 of this thesis.

Signature

 31-1-96

Structural Requirements of 5S rRNA for Nuclear Transport, 7S Ribonucleoprotein Particle Assembly, and 60S Ribosomal Subunit Assembly in *Xenopus* Oocytes

LIZABETH A. ALLISON,^{1*} MELANIE T. NORTH,¹ KIRSTIE J. MURDOCH,¹ PAUL J. ROMANIUK,²
STÉPHANE DESCHAMPS,^{3†} AND MARC LE MAIRE^{3†}

Department of Zoology, University of Canterbury, Christchurch, New Zealand¹; Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia V8W 3P6, Canada²; and Centre de Génétique Moléculaire, Laboratoire propre du Centre National de la Recherche Scientifique, associé à l'Université P. et M. Curie (Paris VI), F-91198 Gif-sur-Yvette Cedex, France³

Received 4 May 1993/Returned for modification 7 July 1993/Accepted 18 August 1993

Structural requirements of 5S rRNA for nuclear transport and RNA-protein interactions have been studied by analyzing the behavior of oocyte-type 5S rRNA and of 31 different in vitro-generated mutant transcripts after microinjection into the cytoplasm of *Xenopus* oocytes. Experiments reveal that the sequence and secondary and/or tertiary structure requirements of 5S rRNA for nuclear transport, storage in the cytoplasm as 7S ribonucleoprotein particles, and assembly into 60S ribosomal subunits are complex and nonidentical. Elements of loops A, C, and E, helices II and V, and bulged and hinge nucleotides in the central domain of 5S rRNA carry the essential information for these functional activities. Assembly of microinjected 5S rRNA into 60S ribosomal subunits was shown to occur in the nucleus; thus, the first requirement for subunit assembly is nuclear targeting. The inhibitory effects of ATP depletion, wheat germ agglutinin, and chilling on the nuclear import of 5S rRNA indicate that it crosses the nuclear envelope through the nuclear pore complex by a pathway similar to that used by karyophilic proteins.

The orchestration of ribosome biogenesis in eukaryotic cells is a process that requires transfer of macromolecules into and out of the nucleus. In *Xenopus* oocytes, 5S rRNA is shuttled between the nuclear and cytoplasmic compartments of the oocyte during different stages of oogenesis in a complex pathway involving different protein associations. In previtellogenic oocytes, 5S rRNA is synthesized before other components of ribosomes are available, is exported from the nucleus, and stored in the cytoplasm as 7S ribonucleoprotein particles (RNPs) (5S rRNA complexed with transcription factor IIIA [TFIIIA]) or as 42S RNPs (5S rRNA complexed with other nonribosomal proteins and tRNA). During vitellogenesis, the 5S rRNA is released from storage and a 5S rRNA-ribosomal protein L5 complex, which is a precursor to assembly into the 60S large ribosomal subunit, forms (reference 3 and references therein). We are interested in the mechanisms that govern the subcellular trafficking of 5S rRNA within the oocyte, particularly the requirements for the mobilization of stored 5S rRNA during ribosome assembly.

There is ample evidence that transit of RNA and RNPs into and out of the nucleus occurs exclusively via the nuclear pores (20, 45; for a review, see reference 30). Although nuclear localization of proteins has been well characterized (1, 10, 21, 49; for a review, see reference 61), the factors governing nuclear export and import of RNA and RNPs remain enigmatic. Nuclear export of tRNA (75), pre-small

nuclear RNAs (50), mRNA (16), 40S and 60S ribosomal subunits (6, 38), and 5S rRNA (24) occurs in a manner consistent with a mediated process. Analysis of the nuclear transport of U small nuclear RNAs has multiple defined, kinetically distinct targeting pathways (27, 47, 48). Nuclear transport of different classes of RNA may thus involve targeting to the pore complex by different cytoplasmic receptors and then translocation into the nucleus by the same pore complex-mediated mechanism. Specific RNA structures have been implicated as requirements for both nuclear import and export (5, 22, 32, 33, 67, 75). Translocation of RNA molecules across the nuclear envelope may also require interaction with specific proteins (28, 31, 33, 43, 62).

RNA-protein interactions are important for many regulatory processes. There is growing evidence that RNA structures, such as helices, loops, bulges, mismatches, and pseudoknots, are key elements in protein recognition (for a review, see reference 19). The sequence and structural requirements for binding of TFIIIA to 5S rRNA have been the subjects of extensive research (7, 8, 56, 66, 74). In comparison, the structural elements of the 5S rRNA molecule required for assembly into ribosomes and for its functional activity within the ribosome remain to be elucidated (34, 37, 69).

We report here the results of a structural analysis of 31 different 5S rRNA mutants by nuclear transport and RNP assembly assays in *Xenopus* oocytes. The results are discussed in relation to the abilities of these same 5S rRNA mutants to bind TFIIIA in vitro (7, 8, 56, 74). We show that determinants of nuclear transport, TFIIIA binding, and ribosome incorporation within the 5S rRNA molecule are complex and nonidentical. The results suggest that TFIIIA binding is not a prerequisite for nuclear targeting of 5S rRNA, consistent with earlier studies indicating that 7S RNPs are not imported (3, 44). We show that microinjected

* Corresponding author. Electronic mail address: l.allison@csc.canterbury.ac.nz.

† Present address: Section de Biophysique des Protéines et des Membranes, Département de Biologie Cellulaire et Moléculaire, Commissariat à l'Énergie Atomique et Centre National de la Recherche Scientifique URA 1290, CE Saclay, F-91191 Gif-sur-Yvette Cedex, France.

5S rRNA is incorporated into 60S ribosomal subunits in the nucleus; thus, the first requirement for assembly is nuclear import. Finally, we demonstrate that nuclear import of 5S rRNA is sensitive to general inhibitors of nuclear pore-mediated translocation.

MATERIALS AND METHODS

Synthesis of mutant 5S rRNAs. The 5S rRNA genes used in these experiments were constructed from a series of synthetic oligonucleotides that were subsequently introduced into pUC18 as previously described (56, 58). Internally labelled 5S rRNAs were produced by *in vitro* transcription from these gene templates with T7 RNA polymerase (Boehringer Mannheim N.Z. Ltd., Auckland, New Zealand) and 50 to 100 μ Ci of [α - 32 P]GTP (3,000 Ci/mmol; Amersham Australia Pty Ltd., Auckland, New Zealand). The mixture was incubated for 1.5 h at 37°C and then treated with RNase-free DNase (Boehringer Mannheim). The sample was extracted with phenol and chloroform, and the RNA transcripts were precipitated twice with 2.5 M ammonium acetate and ethanol. The RNA pellet was resuspended in TE (10 mM Tris-HCl, 1 mM EDTA), pH 7.6, and stored at -80°C. RNA concentration was quantified by DNA Dipstick (Invitrogen Corp., San Diego, Calif.), according to the manufacturer's instructions.

Microinjection and analysis of nuclear transport. An ovary lobe was surgically removed from an adult *Xenopus laevis* (reared at the University of Canterbury on a diet of cockroaches), and the oocytes were separated by brief collagenase treatment as previously described (3). Stage 5-6 oocytes were microinjected with 40 to 80 nl of RNA (0.2 to 0.5 ng of RNA per oocyte) into the oocyte cytoplasm with a PV 830 PicoPump (World Precision Instruments, Inc., New Haven, Conn.) by previously published procedures (3). After overnight incubation (18 h) in O-R2 medium (3), nuclei were manually dissected from oocytes in 1% trichloroacetic acid and collected for analysis (3). RNA was extracted from nuclear and cytoplasmic fractions as described by Allison et al. (3). RNA was analyzed by 8% polyacrylamide-8 M urea gel electrophoresis as described previously (3). Dried gels were autoradiographed on Amersham Hyperfilm-MP at -80°C. A Kontron Uvikon 860 spectrophotometer equipped with a gel scan accessory was used to measure the intensity of bands on suitable exposures of autoradiograms (within the linear range of signal intensity of the film); data were quantified with software package 8543. The system has high resolution, and the software accommodates background levels and irregularly shaped bands. Only mutants that showed less than 50% nuclear transport relative to oocyte-type 5S rRNA were considered to be significantly impaired.

For ATP depletion assays, oocytes were preinjected with 50 nl of 1-U/ μ l apyrase (grade VIII; Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline (PBS) (3) to give a final intracellular concentration of 100 U/ml. Alternatively, 50 nl of PBS was preinjected as a control. After 30-min incubation at 18°C, oocytes were injected with labelled RNA. After an additional 6-h incubation, oocytes were analyzed for nuclear import as described above. ATP depletion was verified with a CLS ATP bioluminescence kit (Boehringer Mannheim). Single oocytes were homogenized in boiling 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.5, and incubated for 5 min at 100°C, and samples were then diluted 1:500 in double-distilled water. A 500- μ l sample was added to an equal volume of luciferase extract immediately prior to measure-

ment of luminescence with an SA1 ATP photometer (model 2000).

Wheat germ agglutinin (WGA) (Sigma Chemical Co.) was dissolved in PBS at concentrations ranging from 0.25 to 2 mg/ml. WGA (50 nl) or PBS (as a control) was injected into the oocyte cytoplasm. After incubation for 3 h, oocytes were injected with labelled RNA and analyzed for nuclear import as described above. As a control for the specificity of preinjection of 50 nl of 1-mg/ml WGA, 50 nl of 1-mg/ml WGA and 500 mM *N*-acetylglucosamine (Sigma Chemical Co.) were coinjected into oocytes prior to injection of labelled 5S rRNA. As a control for nonselective diffusion, oocytes were injected with 50 nl of 100-mg/ml fluorescein isothiocyanate-dextran (molecular weight, 10,000 or 150,000; Sigma Chemical Co.). After overnight incubation, nuclei were dissected out in nucleus isolation buffer (3) and viewed by fluorescence microscopy for diffusion of the dextran into the nucleus.

Since there can be variability in synthetic activity between different batches of oocytes, experiments were repeated a minimum of two times with oocytes from different animals.

Antisera. The antibodies raised against TFIIIA have been shown to react with the relevant protein; no cross-reaction with any other protein was noted by immunoblotting (70). Preparation of the antibodies raised against *Xenopus* 60S ribosomal subunits is described by Viel et al. (70); however, there is a slight difference in the reaction of the anti-60S ribosomal subunit antiserum used in this study (anti-serum number 2679; sampling date, 7 November 1985) and of the antiserum described by Viel et al. (70). Immunoblot analysis was performed on total protein from ribosomal subunits purified from mature *Xenopus* ovaries by ultracentrifugation techniques (70) or from 7S fractions obtained after sucrose density centrifugation of cell homogenates of *Xenopus* ovaries (41). The proteins were fractionated by polyacrylamide gel electrophoresis and either stained with Coomassie blue or transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore S.A., Saint-Quentin Yvelines, France). Membranes were incubated with diluted antiserum (1:287) in a solution of 10 mM sodium phosphate (pH 7.4), 150 mM NaCl, and 1% bovine serum albumin, washed in the same buffer, treated with 35 S-labelled-protein A in order to label the antigen-antibody complexes, washed again, and exposed to X-ray films at -80°C by previously published procedures (70).

To further ensure that the anti-60S antiserum did not cross-react with 7S RNPs, immunoprecipitation analyses were performed on 7S RNPs purified from immature *Xenopus* ovaries as previously described (3). Immunoprecipitation assays were performed as described by Allison et al. (3), using approximately 10 μ g of 7S RNPs and 10 μ l of antiserum bound to protein A-Sepharose (Pharmacia LKB Biotechnology, Auckland, New Zealand). Immunoprecipitates were vortexed briefly in sample buffer (2% sodium dodecyl sulfate, 100 mM dithiothreitol, 60 mM Tris [pH 6.8], 0.01% bromophenol blue), heated at 100°C for 5 min, and then centrifuged for 30 s. Proteins from the supernatant were resolved by polyacrylamide gel electrophoresis and silver stained with a Bio-Rad silver stain kit (Bio-Rad Laboratories Pty Ltd., Auckland, New Zealand), according to the manufacturer's instructions.

Immunoprecipitation assays. Microinjected oocytes were incubated for 48 h at 18°C. Immunoprecipitation assays were performed as described by Allison et al. (3), using homogenates of 20 microinjected oocytes and 10 μ l of antiserum bound to protein A-Sepharose. For nuclear immunoprecipitations, 20 nuclei were isolated in nucleus isolation buffer

(3). RNA was recovered from the immunoprecipitates and immunosupernatants and resolved on 8% polyacrylamide-8 M urea gels, and then autoradiography was performed. Results were interpreted qualitatively by comparing the amounts of the different mutants immunoprecipitated with the amount of oocyte-type 5S rRNA immunoprecipitated and by comparing the relative specific activities of the RNA mutants and the amounts of RNA injected, using the supernatant fractions.

Electrophoretic mobility shift assays. After a 48-h incubation at 18°C, five microinjected oocytes were homogenized in 20 μ l of RNP homogenization buffer (20 mM Tris [pH 7.6], 100 mM KCl, 1.5 mM MgCl₂, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 U of RNasin per ml; Promega-Pacific Diagnostics Pty. Ltd., Auckland, New Zealand). A crude cellular lysate was prepared by disrupting oocytes with a Gilson tip, spinning down the cellular debris (yolk and pigment) by centrifugation at $9,000 \times g$ for 10 min at 4°C, and removing the supernatant, taking care not to disturb the lipid pellicle floating on the surface. Glycerol-dye loading buffer was added to the supernatant, and samples were loaded directly onto a 6% polyacrylamide-0.1% Triton X-100 gel in Tris-borate-EDTA buffer plus 0.1% Triton X-100. Samples were electrophoresed at 300 V; gels were dried at 80°C for 30 min, and then autoradiography was performed. A sample of unlabelled, native 7S RNPs were included as a marker. The marker lane was stained with ethidium bromide and viewed by UV illumination.

RESULTS

Sequence and structural requirements for nuclear transport of 5S rRNA. The sequence and structural requirements of 5S rRNA for nuclear transport in *Xenopus* oocytes were investigated by analyzing 31 mutant 5S rRNA molecules generated by in vitro transcription. Figure 1 shows the secondary structure of oocyte-type 5S rRNA and the locations of the mutations analyzed. With the exception of seven mutants, substitutions were located between nucleotides 11 and 108 in the region of the RNA molecule shown to provide the necessary sequence and conformational information required for nuclear transport (3). In our previous study, however, we did not investigate quantitative differences in nuclear transport.

All mutant 5S rRNAs were stable 24 h after cytoplasmic microinjection (data not shown). Analysis of oocyte-type 5S rRNA revealed that on average, 14% of the microinjected RNA was found in the nucleus after 18 h (Fig. 2, lanes 1 and 2). These results were consistent with a previous study, which demonstrated that the total amount of labelled 5S rRNA found in the nucleus increases for up to 18 to 21 h and then remains constant (3). Presumably, this constant amount reflects steady-state levels of 5S rRNA molecules which have migrated into the nucleus and are being assembled into ribosomes. Nuclear transport of 5S rRNA thus represents a state of flux between the cytoplasm and nucleus, as opposed to a measure of nuclear import alone. The 31 mutant 5S rRNAs showed a variety of transport phenotypes. The intracellular distribution of five mutant RNAs is presented in Fig. 2. The most defective mutant, 16-21, showed a 75% reduction in nuclear transport relative to that of oocyte-type 5S rRNA (lanes 3 and 4). Mutants 41-44 and 105-108 were less defective in nuclear transport, showing reductions of 67 and 54%, respectively (compare lanes 5 and 6 and lanes 11 and 12). In contrast, mutants Δ 63 and 67-70 had transport

characteristics similar to those for oocyte-type 5S rRNA (lanes 7 to 10). The data are summarized in Table 1.

Mutations within predicted helices II and V tend to have lower nuclear transport values if the mutations are helix breaking (HB in Table 1), than if the mutations are helix maintaining (HM). Nucleotide substitutions that alter either the 5' sequence of helix II or the sequence and structure of helix V resulted in a significant reduction ($\geq 50\%$) in nuclear transport. The loop mutants tested have block sequence substitutions in one loop which alter the sequence but maintain the single-stranded secondary structure (56). Nucleotide substitutions that alter the 3' sequence of loop C or the noncanonical base pairing in loop E (57, 72) resulted in a significant reduction in nuclear transport. The deletion of bulged nucleotides did not impair transport. In contrast, alteration of the hinge nucleotide at position 66 resulted in a 57% reduction in nuclear transport, suggesting this nucleotide is located in a key region of the 5S rRNA. This is in line with a previous study showing that nucleotides in the hinge region of the 5S rRNA, the junction of the three helical domains, play a central role in determining the coaxial stacking interactions and tertiary structure of the RNA and are critical for TFIIIA recognition (8). In summary, specific structural elements of the 5S rRNA molecule have been shown to be important for nuclear transport, but no single region of 5S rRNA is solely responsible.

TFIIIA has been implicated in mediating 5S rRNA nuclear export (31), but indirect evidence suggests that 7S RNPs are not import competent (3, 44). In order to determine whether there is a correlation between wild-type TFIIIA binding affinity and wild-type nuclear transport, results on nuclear transport of the mutants are compared with data on their ability to bind TFIIIA in vitro from previous studies (7, 8, 56, 74). In general, the two sets of data are comparable (Table 1); there are, however, important exceptions. For example, mutants 10-13, 57-62, and G109 possess low binding affinities for TFIIIA (17 to 40% of oocyte-type 5S rRNA), yet the measure of nuclear transport is closer to that of oocyte-type 5S rRNA (63 to 77%). Furthermore, other mutants (e.g., 96-101 and 14-15) with higher TFIIIA binding affinities (59 to 85%) exhibit defective nuclear transport (36 to 49%). Since some of the structural requirements of 5S rRNA for nuclear transport and 7S RNP assembly differ, this suggests that TFIIIA binding is not a prerequisite for nuclear targeting.

Nuclear import of 5S rRNA and mutant RNA molecules occurs by a mediated process. One possible explanation for oocyte-type and mutant 5S rRNAs entering the nucleus is that their small size allows for unrestricted entry. 5S rRNA is a 121-nucleotide molecule of approximately 41 kDa. The nuclear pore complex contains an aqueous channel of 9- to 11-nm diameter which acts like a molecular sieve; this channel allows rapid, nonselective diffusion of molecules of approximately 20 to 40 kDa, while excluding larger cytoplasmic proteins (52). A truncated, 98-nucleotide 5S rRNA does not enter the nucleus (Fig. 2) (3), however, which implies that nuclear uptake of full-length 5S rRNA occurs by a mechanism other than unrestricted entry. Further, comparing 5S rRNA and a globular protein may not be a valid comparison; the frictional ratio of naked 5S rRNA has been shown to be higher than that of 7S RNPs, suggesting that 5S rRNA is more elongated (17).

Translocation across the nuclear envelope occurs by an energy-dependent process (2, 51, 55). Thus, sensitivity to ATP depletion is one criterion for distinguishing active transport from diffusion. We therefore tested the effect of cytoplasmic ATP depletion on 5S rRNA nuclear import.

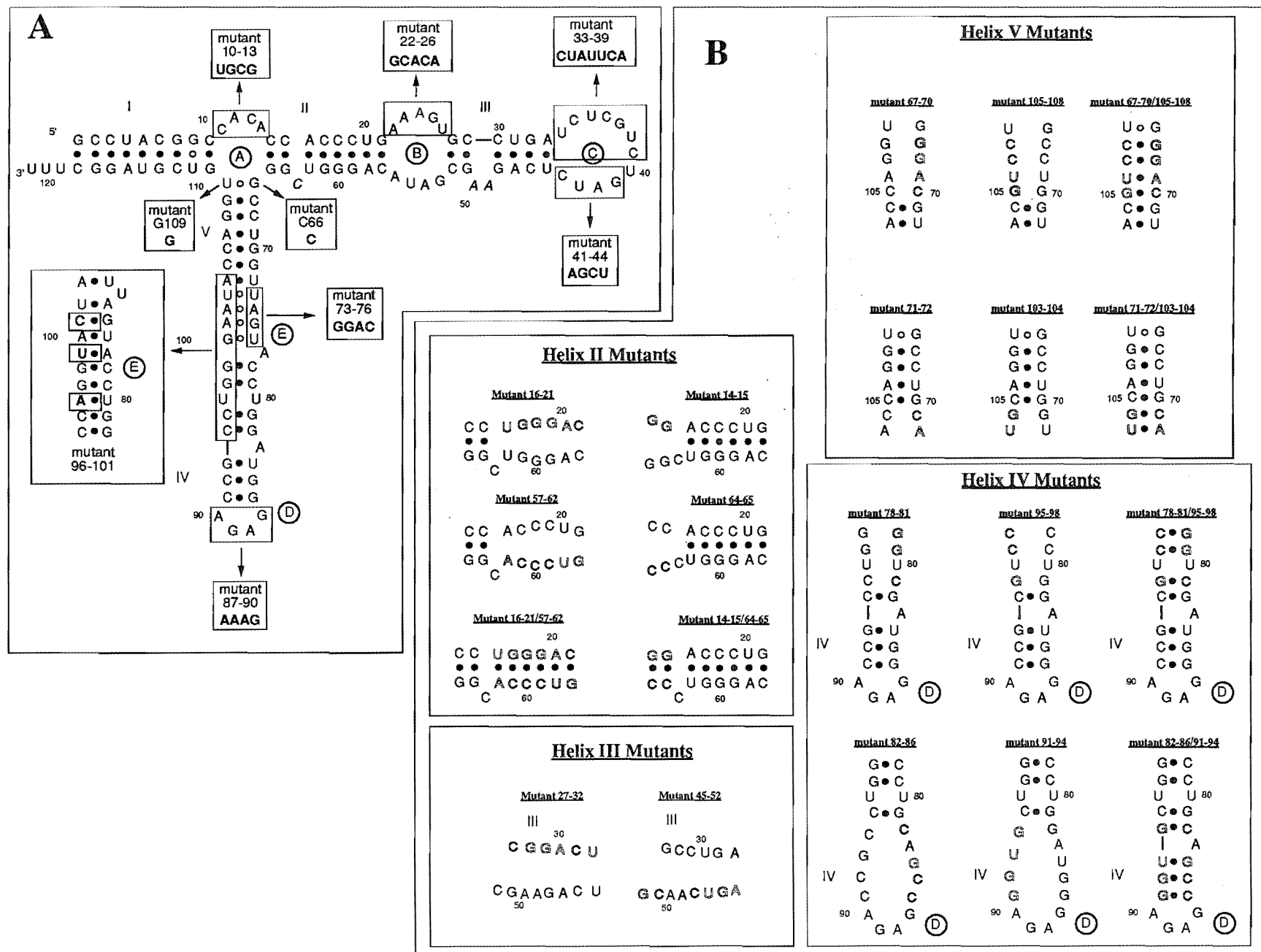


FIG. 1. Secondary structure of *X. laevis* oocyte-type 5S rRNA showing mutant nucleotide substitutions and deletions. (A) Single-strand substitution mutations. The bulged nucleotides deleted (A at nucleotides 49 and 50 and C at nucleotide 63) are indicated by italicized letters. (B) Helix mutants. Only the relevant region of the 5S rRNA is shown. Nucleotide substitutions are indicated by outlined letters.

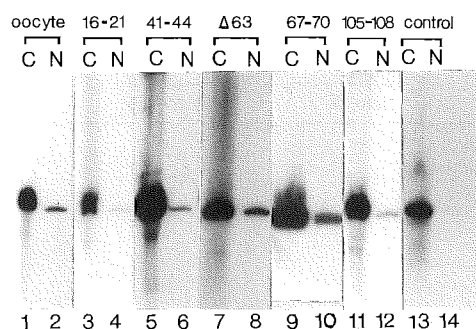


FIG. 2. Intracellular distribution of ^{32}P -labelled oocyte-type or mutant 5S rRNA species following microinjection in *X. laevis* oocytes. ^{32}P -labelled oocyte-type or mutant 5S rRNA was microinjected into the cytoplasm of fully grown oocytes. After overnight (18-h) incubation, RNA was extracted from five pooled nuclear (N) and cytoplasmic (C) fractions and separated electrophoretically on denaturing 8% polyacrylamide gels containing 8 M urea. The numbers refer to those nucleotides in the oocyte-type 5S rRNA (oocyte) which have been substituted or deleted (Fig. 1). A truncated 5S rRNA molecule consisting of nucleotides 1 to 98 (control) which does not enter the nucleus was included as a control for isolation of nuclei free of cytoplasmic contamination.

After injection of the ATP-hydrolyzing enzyme apyrase, ATP depletion was verified by a luciferase assay. At a final intracellular concentration of 100 U/ml, there was a rapid decline in ATP levels from 2 mM to 10 to 40 μM . This decrease occurred within 30 min and remained low after 25 h of incubation (data not shown). As shown in Fig. 3, ATP depletion significantly inhibited nuclear import of oocyte-type 5S rRNA (lane 4). The autoradiogram was deliberately overexposed to demonstrate the absence of detectable levels of labelled 5S rRNA in the nuclear fraction.

Another criterion for signal-mediated import via the nuclear pore complex is sensitivity to the lectin WGA. WGA is known to inhibit active transport by binding to *N*-acetylglucosamine (GlcNAc)-containing proteins present in the nuclear pore complex (25, 26, 65). Preinjection of WGA significantly inhibited nuclear import of oocyte-type 5S rRNA (Fig. 4A, lane 4). To verify that inhibition of import resulted from WGA interacting with GlcNAc-containing glycoproteins, WGA was coinjected with GlcNAc. Figure 4A (lane 6) shows that the presence of 50 mM GlcNAc abolished WGA-induced inhibition of import. The inhibitory effect of preinjected WGA on oocyte-type 5S rRNA was dose dependent (Fig. 4B), with 86% inhibition occurring at a concentration of 0.1 mg/ml. To ensure that WGA was not acting by physically occluding the nuclear pores, small fluorescently labelled dextrans (10 kDa) were microinjected into the oocyte cytoplasm. After overnight incubation, oocytes were dissected, and the nuclei were viewed by fluorescence microscopy. Preinjection of WGA had no effect on nonselective diffusion of small dextrans, and large fluorescently labelled dextrans (150 kDa) were excluded from the nucleus (data not shown). Similar results have been obtained in cultured somatic cells (15, 25).

An additional criterion for mediated transport is exclusion of nuclear proteins from the nucleus in chilled cells. Nuclear accumulation of large and small karyophilic proteins is inhibited by chilling, which slows enzyme-mediated reactions, whereas small proteins lacking nuclear localization signals may diffuse freely across the nuclear envelope, regardless of the temperature (10). Thus, 5S rRNA nuclear

import was tested for temperature dependence. Figure 5 shows that the nuclear import of oocyte-type 5S rRNA was inhibited in chilled oocytes (lane 4). Chilling did not inhibit nonselective diffusion of a small fluorescent dextran into the nucleus (data not shown).

To determine whether nuclear import of mutant 5S rRNAs occurs by a mediated process or by nonselective diffusion, loop C mutant 41-44 and loop E mutant 96-101 were tested, since these RNAs have reduced abilities to enter the nucleus relative to that of oocyte-type 5S rRNA (Table 1). Preinjection of WGA significantly inhibited nuclear import of both mutant 5S rRNAs, although mutant 41-44 was less sensitive. Data are summarized in Table 2. Nuclear import of mutants 41-44 and 96-101 was also inhibited in chilled oocytes (Table 2), although both mutants were less sensitive than oocyte-type 5S rRNA. The possibility remains that nuclear entry of these mutants is partly diffusional; binding to an import receptor protein may be of low affinity or weakly temperature sensitive. Not all RNA-protein interactions, however, are temperature sensitive, because assembly of microinjected 5S rRNA into 7S RNPs (storage particles) does occur in chilled oocytes (data not shown).

In summary, nuclear import of 5S rRNA is sensitive to general inhibitors of nuclear pore-mediated translocation, so import is likely accomplished by a pathway similar to that used by karyophilic proteins.

5S rRNA is assembled into 60S ribosomal subunits in the nucleus. Although indirect evidence to date suggests that cytoplasmic 5S rRNA returns to the nucleus for ribosome incorporation (3, 18), a nuclear assembly site has not yet been demonstrated. To deduce the site of 5S rRNA assembly into 60S ribosomal subunits, labelled 5S rRNA was microinjected into the cytoplasm and subsequently immunoprecipitated from the nuclear versus cytoplasmic compartment.

The anti-60S ribosomal subunit serum detects two major bands in extracts of 60S ribosomal subunits (Fig. 6A and B, lanes 3); one band is approximately 37 kDa, and the other is approximately 14 to 15 kDa. The 37-kDa band corresponds to ribosomal protein L2; the 14- to 15-kDa band is not as easily identified but migrates similarly to ribosomal proteins in the range of L17 to L20 (53). This antiserum also reacts slightly with an approximately 18-kDa band in extracts of 40S ribosomal subunits (Fig. 6A and B, lanes 4). In the 7S fraction from a sucrose gradient, apart from the high-molecular-mass bands which probably represent polysaccharides, there is no cross-reaction (Fig. 6A and B, lanes 2). This antiserum does not immunoprecipitate 7S RNPs (Fig. 6C, lane 2). Intact ribosomes are immunoprecipitated by the anti-60S serum; the immunoprecipitates contain proteins that react with anti-40S ribosomal subunit serum as revealed by immunoblotting and contain both 28S and 18S rRNA as revealed by Northern blot (RNA) analysis (data not shown).

60S subunits containing labelled 5S rRNA were first faintly detectable in the nuclear fraction after 18 h but were not detectable in the cytoplasm (data not shown). After 48 h, labelled 5S rRNA was immunoprecipitated as 60S subunits from both the cytoplasmic fraction (Fig. 7, lane 2) and the nuclear fraction (lane 4), thus indicating a nuclear site of assembly, followed by export to the cytoplasm.

In a previous study, 7S RNPs were detected only in the oocyte cytoplasm after microinjection of labelled 5S rRNA and immunoprecipitation with anti-TFIIIA antibodies (3). Since TFIIIA has been shown to bind nuclear 5S rRNA (31), we repeated these assays with a different antibody preparation to determine whether a small nuclear pool of 7S RNPs was present. The antibodies raised against TFIIIA react with

TABLE 1. Summary of mutant phenotypes

Mutant designation ^a	Nuclear transport relative to oocytes type ^b	TFIIIA K_a in vitro ^c	Immunoprecipitation ^d		7S RNP EMSA ^e
			Anti-7S	Anti-60S	
Oocyte type	1.00	1.00	+	+	+
Helix II					
14-15 (HB)	0.49 ± 0.03 (3)	0.85 ± 0.22	IR	IR	+
64-65 (HB)	0.97 (1)	0.74 ± 0.24	—	+	+
14-15/64-65 (HM)	1.04 ± 0.16 (3)	1.11 ± 0.32	IR	+	+
16-21 (HB)	0.25 ± 0.05 (2)	0.32 ± 0.15	+	IR	ND
57-62 (HB)	0.73 (1)	0.40 ± 0.15	+	+	+
16-21/57-62 (HM)	1.88 (1)	1.09 ± 0.48	+	+	+
Helix III					
27-32 (HB)	0.70 ± 0.23 (2)	0.75 ± 0.10	+	+	+
45-52 (HB)	1.45 ± 0.34 (3)	0.76 ± 0.12	+	+	+
Helix IV					
78-81 (HB)	0.99 (1)	0.88 ± 0.01	+	+	ND
95-98 (HB)	0.86 (1)	0.78 ± 0.02	+	IR	+
78-81/95-98 (HM)	1.19 ± 0.46 (3)	0.86 ± 0.01	+	+	ND
82-86 (HB)	1.75 ± 0.02 (2)	0.81 ± 0.30	+	+	ND
91-94 (HB)	0.85 ± 0.31 (4)	0.96 ± 0.18	+	+	+
82-86/91-94 (HM)	1.38 ± 0.19 (2)	1.21 ± 0.35	+	+	ND
Helix V					
67-70 (HB)	1.18 ± 0.44 (2)	0.75 ± 0.12	—	+	+
105-108 (HB)	0.46 ± 0.15 (4)	0.39 ± 0.06	+	+	ND
67-70/105-108 (HM)	1.07 ± 0.34 (2)	0.71 ± 0.01	+	IR	+
71-72 (HB)	0.44 ± 0.12 (3)	0.35 ± 0.21	+	IR	ND
103-104 (HB)	0.48 ± 0.18 (3)	0.50 ± 0.23	+	IR	ND
71-72/103-104 (HM)	1.37 ± 0.43 (3)	1.18 ± 0.32	+	+	ND
Loop A					
10-13 (SEQ)	0.63 ± 0.00 (2)	0.30 ± 0.01	—	—	—
Loop B					
22-26 (SEQ)	1.18 (1)	1.00 ± 0.02	+	+	+
Loop C					
33-39 (SEQ)	0.97 ± 0.25 (3)	1.00 ± 0.02	+	+	+
41-44 (SEQ)	0.33 ± 0.06 (3)	0.40 ± 0.10	+	+	+
Loop D					
87-90 (SEQ)	1.26 (1)	0.71 ± 0.10	+	+	+
Loop E					
73-76 (SEQ)	1.60 ± 0.20 (2)	0.57 ± 0.02	—	+	+
96-101 (SEQ)	0.36 ± 0.04 (3)	0.59 ± 0.01	—	—	+
Bulged nucleotides					
Δ49,50 (DEL)	0.76 ± 0.38 (2)	1.00 ± 0.02	+	—	+
Δ63 (DEL)	1.04 ± 0.40 (4)	1.00 ± 0.02	+	—	+
Hinge nucleotides					
C66 (SEQ)	0.43 ± 0.05 (2)	0.12 ± 0.01	+	+	+
G109 (SEQ)	0.77 ± 0.02 (2)	0.17 ± 0.05	—	+	+

^a The numbers in the designations refer to those nucleotides in 5S rRNA which have been substituted or deleted (Fig. 1). HB, helix breaking; HM, helix maintaining; SEQ, sequence mutation; DEL, deletion.

^b Assays were performed as described in the legend to Fig. 2; nuclear transport was quantified by densitometry of suitable exposures of autoradiograms (within the linear range of signal intensity of the film), relative to the steady-state levels of oocyte-type 5S rRNA localized to the nucleus during each experiment. Experimental values are presented (mean ± standard deviation from the mean). Numbers in parentheses indicate the number of independent experiments performed.

^c In vitro binding data is summarized from references 7, 8, 56, 58, and 74.

^d Assays were performed as described in the legend to Fig. 8A. Protein binding was quantified by qualitative estimation of relative amounts of bound and free RNA. IR, inconclusive results caused by RNA with low specific activity.

^e Electrophoretic mobility shift assays (EMSAs) were performed as described in the legend to Fig. 8C. ND, not determined.

the relevant protein (Fig. 6C, lane 1); no cross-reaction was noted with any other protein by immunoblotting (70). Under similar conditions, in this present study 7S RNPs containing labelled 5S rRNA were detectable only in the cytoplasmic fraction from 10 oocytes incubated for 21 h after microinjection

(data not shown). However, a small nuclear pool of 7S RNPs was revealed by increasing the incubation time after microinjection to 48 h, increasing the specific activity of the labelled 5S rRNA, and increasing the sample size to 20 oocytes (Fig. 7, lane 3).

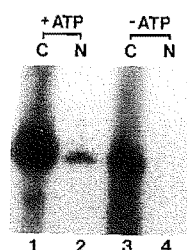
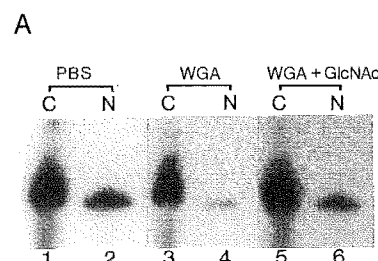


FIG. 3. Nuclear import of oocyte-type 5S rRNA is ATP dependent. Oocytes were preinjected with a final intracellular concentration of 100 U of apyrase per ml ($-ATP$), or PBS as a control ($+ATP$). After incubation for 30 min at $18^{\circ}C$, ^{32}P -labelled oocyte-type 5S rRNA was injected into the cytoplasm, and the oocytes were incubated for a further 6 h. The analysis for nuclear import was then performed as described in Fig. 2. C, cytoplasm; N, nucleus.

Sequence and structural requirements of 5S rRNA for 60S ribosomal subunit assembly and 7S RNP assembly in vivo. Since the results above demonstrated that the first requirement for subunit assembly is nuclear targeting, it was of interest to ascertain which sequence and structural elements of 5S rRNA are required for incorporation into 60S ribosomal subunits. Immunoprecipitation assays were thus performed on microinjected oocytes with antibodies against 60S subunits.

The results of several representative experiments are shown in Fig. 8A, and the complete data are presented in Table 1. Of 31 mutants, only 4 were completely defective in ribosome incorporation. Of these four mutants, two had mutations in single-strand loop regions, mutants 96–101 (Fig. 8A, lane 14) and 10–13 (lane 16), indicating that these regions of the molecule are important in recognition or binding of 5S rRNA with other ribosomal components. Comparable amounts of oocyte-type and mutant 5S rRNAs were detectable in the immunosupernatant fractions, demonstrating that the mutants were not degraded during the 48-h incubation (Fig. 8B). The simplest explanation for mutant 96–101 not being detected in ribosomes is that this results from impaired nuclear transport (Table 1). However, mutant 41–44 is equally defective in nuclear transport, yet it is found incorporated into 60S ribosomal subunits (lane 12). The other two defective mutants were $\Delta 49,50$ (lane 18) and $\Delta 63$ (lane 20), indicating that these bulged nucleotides are important for 5S rRNA incorporation into the 60S subunit. Results were inconclusive for six mutants (Table 1). The possibility that the absence of these labelled RNAs in 60S ribosomal subunits was due to the low specific activity of the RNA could not be ruled out. Despite repeated attempts to increase the specific activity, these particular mutant 5S rRNA gene constructs continued to provide poor templates for transcription.

In addition to assaying different mutant 5S rRNA molecules for their incorporation into 60S subunits, immunoprecipitation of 7S RNPs was also performed with anti-TFIIIA antibodies. This qualitative analysis provided a comparison with the *in vitro* data previously obtained for TFIIIA binding to the mutant 5S rRNA molecules (summarized in Table 1). All but six mutant 5S rRNAs were found to be able to bind TFIIIA in the oocyte in an immunodetectable complex. One of these defective mutants, 10–13 (Fig. 8A, lane 15), corresponds with the *in vitro* data; mutant 10–13 has a K_d of 0.30 and is 20 times less effective in inhibiting TFIIIA binding than oocyte-type 5S rRNA is (56). Negative results with other mutants, however, were contradictory. For example,



B

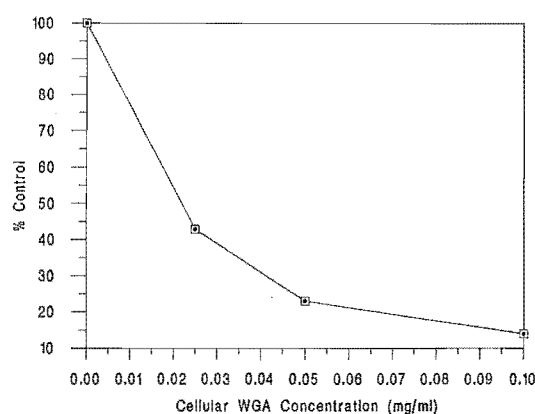


FIG. 4. Nuclear import of oocyte-type 5S rRNA is inhibited by WGA. (A) Oocytes were preinjected with WGA at a final intracellular concentration of 0.1 mg/ml or with WGA (0.1 mg/ml) and 50 mM *N*-acetylglucosamine (WGA + GlcNAc). PBS was preinjected as a control. After incubation for 3 h, ^{32}P -labelled oocyte-type 5S rRNA was injected into the cytoplasm and the oocytes were incubated for a further 18 h. The analysis for nuclear import was then performed as described in the legend to Fig. 2. C, cytoplasm; N, nucleus. (B) Dose-dependent inhibition of 5S rRNA nuclear import. Oocytes were preinjected with 50 nl of WGA at various concentrations. The final intracellular concentration of WGA is denoted on the x axis. The nuclear import of ^{32}P -labelled 5S rRNA was assayed 18 h after injection. Import in the presence of WGA is expressed as a percentage of the import of 5S rRNA in control oocytes preinjected with PBS only (% Control).

mutant 67–70 (Fig. 8A, lane 7) was not immunoprecipitated with anti-TFIIIA yet has an *in vitro* binding affinity of 0.75 (74). To clarify these results, homogenates of microinjected oocytes were analyzed by electrophoretic mobility shift assays. As shown in Fig. 8C, mutant 67–70 (lane 5) clearly showed a band shift present at a position corresponding to that of 7S RNPs. Similarly, mutants 64–65, 73–76, 96–101, and G109 which were not immunodetectable by anti-TFIIIA showed band shifts corresponding to 7S RNPs (Fig. 8A and C and Table 1). In contrast, consistent with the *in vitro* binding data and immunoprecipitation data, no band shift corresponding to 7S RNPs was detected with mutant 10–13 (Fig. 8C, lane 8). The data are summarized in Table 1.

The inconsistent anti-TFIIIA immunoprecipitation assay and electrophoretic mobility shift assay results described above can be explained by reasoning that for these mutant 5S rRNA molecules, the 5S rRNA-TFIIIA complexes that are formed have different conformations which are not recognized by the anti-TFIIIA antibody or that the complexes formed are not stable under the assay conditions.



FIG. 5. Effect of chilling on the nuclear import of oocyte-type 5S rRNA. 32 P-labelled oocyte-type 5S rRNA was injected into the oocyte cytoplasm. After incubation for 18 h at either 18°C as a control or on ice (0°C), the analysis for nuclear import was performed as described in the legend to Fig. 2. C, cytoplasm; N, nucleus.

These observations raise a cautionary note toward relying solely on antigen-antibody interactions for the analysis of RNA-protein interactions.

The potential formation of an RNA-protein complex not recognized by an antibody is not a likely consideration with the anti-60S ribosomal subunit antibody. 5S rRNA is thought to be buried in the 60S subunit (42), within the peptidyl transferase center, whereas the ribosomal proteins recognized by this antibody (L2 and another in the range of L17 to L20) are not detected in this region (23, 63, 68). Given the apparent lack of direct cross-links between 5S rRNA and the ribosomal proteins recognized by the anti-60S antibody, it is unlikely that any conformational change resulting from incorporation of a mutant 5S rRNA molecule into the 60S subunit would be transmitted to these ribosomal proteins;

TABLE 2. Summary of effects of WGA and chilling on nuclear import of oocyte-type and mutant 5S rRNA

RNA	% Control ^a	
	WGA	0°C
Oocyte type	14.6 (\pm 0.4)	4.1 (\pm 2.1)
41-44	42.0 (\pm 3.4)	43.0 (\pm 3.0)
96-101	17.0 (\pm 1.6)	22.0

^a Nuclear import of the designated 32 P-labelled RNA (Fig. 1) was assayed in samples of five oocytes as described in the legend to Fig. 4A for WGA (final intracellular concentration of 0.2 mg/ml) and in the legend to Fig. 5 for chilling. Import was quantified by densitometry and expressed as a percentage (\pm standard deviation from the mean) of the amount of import in control oocytes (% Control). Tabulated results are from two samples, except for mutant 96-101 (0°C) for which one sample was used.

thus, the recognition or binding of the anti-60S antibody to these epitopes would not be affected.

In summary, specific structural elements of the 5S rRNA molecule have been shown to be critical for ribosome incorporation. Deletion of bulged nucleotides and nucleotide substitutions that alter the sequence of loop A or the noncanonical base pairing in loop E (57, 72) abolish ribosome incorporation. Some of the structural elements of 5S rRNA required for ribosome incorporation differ from those elements required for nuclear transport. Further, some of the structural requirements of 5S rRNA for 7S RNP assembly and for 60S ribosomal subunit assembly differ.

DISCUSSION

From the 5S rRNA mutants studied in this report, a picture emerges of complex and nonidentical structural features within the central domain of the RNA molecule that

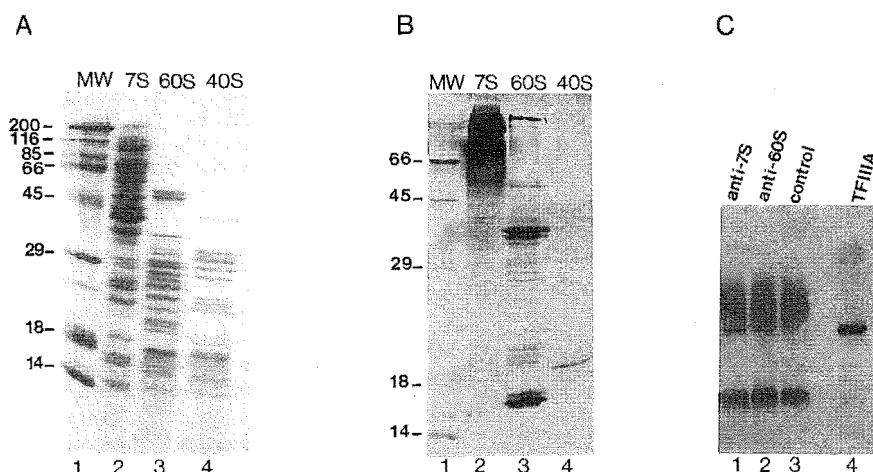


FIG. 6. Control of antiserum specificity by immunoblotting and immunoprecipitation assays. Total protein from 7S fractions and from ribosomal subunits purified from *Xenopus* ovaries was fractionated by electrophoresis in 15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) and either stained with Coomassie blue (A) or transferred to Immobilon-P polyvinylidene difluoride membranes and probed with anti-60S ribosomal subunit antiserum (B). The antigen-antibody complexes were revealed by treatment with 35 S-labelled protein A and then autoradiography (48 h). The high-molecular-mass bands in lane 2 of panel B are probably polysaccharides. Lanes: MW, protein molecular mass markers (in kilodaltons); 7S, approximately 10 μ g of protein purified from the 7S region of a sucrose gradient; 60S, approximately 3 μ g of protein from 60S ribosomal subunits purified by sucrose gradient centrifugation; 40S, approximately 1 μ g of protein from 40S ribosomal subunits purified by sucrose gradient centrifugation. (C) 7S RNPs purified from immature *Xenopus* ovaries were incubated with protein A-Sepharose-antibody complexes in an immunoprecipitation assay. Proteins were recovered and separated electrophoretically on 12% polyacrylamide gels containing 0.1% SDS and then silver stained. Lanes: anti-7S, immunoprecipitation with anti-TFIIIA; anti-60S, immunoprecipitation with anti-60S ribosomal subunit; control, mock immunoprecipitation with anti-60S antiserum, but without 7S RNPs; TFIIIA, TFIIIA protein from 7S RNPs.

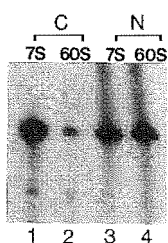


FIG. 7. Intracellular localization of microinjected 5S rRNA in 60S ribosomal subunits and 7S RNPs. Labelled 5S rRNA was microinjected into the cytoplasm of oocytes. After incubation for 48 h, 20 oocytes were manually fractionated into cytoplasmic (C) and nuclear (N) compartments. Homogenates from each compartment were incubated with protein A-Sepharose-antibody complexes in an immunoprecipitation assay. Labelled RNAs were recovered and separated electrophoretically on 8% polyacrylamide gels containing 8 M urea. Lanes: 7S, immunoprecipitation with anti-TFIIIA; 60S, immunoprecipitation with anti-60S ribosomal subunit.

are required for nuclear transport, assembly into 7S RNPs (storage particles) and assembly into 60S ribosomal subunits (Fig. 9). 5S rRNA is assembled into 60S ribosomal subunits in the nucleus; thus, the first requirement for subunit assembly is nuclear targeting. However, some of the structural elements of 5S rRNA required for ribosome incorporation differ from those elements required for nuclear transport (Fig. 9). Differences in the requirements for nuclear transport and TFIIIA binding suggest that TFIIIA binding is not a prerequisite for nuclear targeting of 5S rRNA.

Subcellular trafficking of 5S rRNA. Since 5S rRNA can be easily and specifically dissociated from intact ribosomes or subunits as a 5S rRNA-L5 complex (3), it was thought that the 5S rRNA might be added as a surface component to partially assembled subunits, hence a cytoplasmic site of integration of stored 5S rRNA seemed plausible. However, results presented here indicate that cytoplasmically stored 5S rRNA returns to the nucleus for assembly into the large 60S subunit. This conclusion is consistent with a recent study in *Saccharomyces cerevisiae*, which suggests that 5S rRNA is crucial to an early step in subunit assembly (69). Thus, analysis of intracellular distribution reflects the steady-state levels of 5S rRNA molecules reached after overnight incubation (3); 5S rRNA is released from storage, migrates into the nucleus, is assembled into ribosomes, and then is exported to the cytoplasm. Since ribosome assembly continues for a protracted period of time in *Xenopus* oocytes, only a small fraction of microinjected 5S rRNA would be expected to appear in the nucleus at any given time or be incorporated into nascent ribosomes. The latter is reflected in the small fraction of labelled 5S rRNA found in 60S subunits compared with 7S RNPs (storage particles). Interestingly, a number of mutants accumulated in the nucleus to a greater degree than oocyte-type 5S rRNA, for example, nuclear accumulation of mutant 16-21/57-62 was nearly twice that of oocyte-type 5S rRNA. It is not clear whether this distribution represents enhanced nuclear import or whether the increased nuclear accumulation of some mutants is due to a defect in another function, such as nuclear export.

Mediated nuclear import of 5S rRNA. Nuclear import of 5S rRNA is inhibited by cytoplasmic ATP depletion, WGA, and chilling. Similarly, nuclear protein import (2, 51, 55), export of ribosomal subunits (6), and mRNA export (16) require metabolic energy in vivo. The concentration of WGA re-

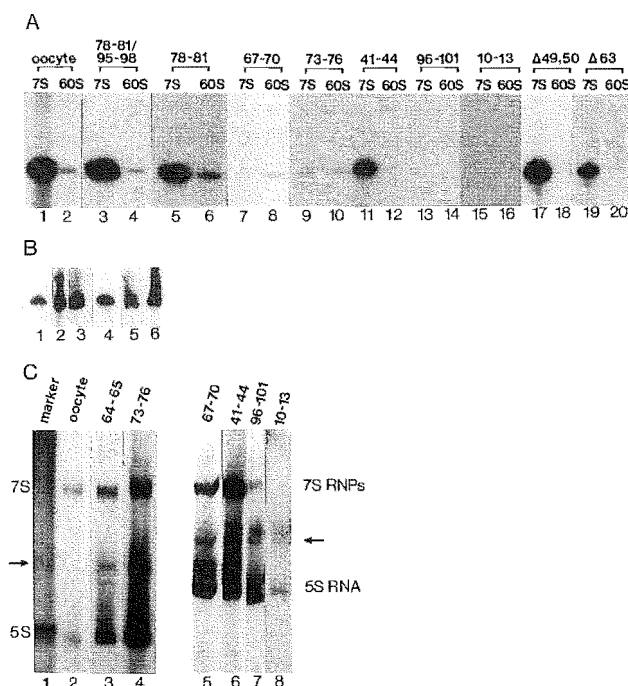


FIG. 8. Assembly of mutant 5S rRNAs into 60S ribosomal subunits and 7S RNPs. (A) 32 P-labelled oocyte-type or mutant 5S rRNA was microinjected into the oocyte cytoplasm. After 48 h, homogenates of whole oocytes were analyzed by immunoprecipitation assay as described in the legend to Fig. 7. Labelled RNAs were recovered and separated electrophoretically on 8% polyacrylamide gels containing 8 M urea. The numbers in the mutant designations refer to those nucleotides in oocyte-type 5S rRNA (oocyte) which have been substituted or deleted (Fig. 1). Lanes: 7S, immunoprecipitation with anti-TFIIIA; 60S, immunoprecipitation with anti-60S ribosomal subunit. (B) Labelled RNAs recovered from immunosupernatant fractions, from the anti-60S immunoprecipitation assays in panel A. Lane 1, oocyte-type RNA; lane 2, mutant 67-70; lane 3, mutant 96-101; lane 4, mutant 10-13; lane 5, mutant Δ49,50; lane 6, mutant Δ63. (C) Electrophoretic gel mobility shift assays of mutant 5S rRNAs. Labelled oocyte-type or mutant 5S rRNA was injected into the oocyte cytoplasm. After 48 h, homogenates were electrophoresed on nondenaturing 6% polyacrylamide gels containing 0.1% Triton X-100. The location of 7S RNPs was determined by running an additional lane of unlabelled, purified 7S RNPs; this lane was stained with ethidium bromide and visualized by UV illumination (not shown). The arrows indicate a band shift that may represent the 5S rRNA-ribosomal protein L5 complex, but this has yet to be confirmed. Marker, 32 P-labelled 5S rRNA. Lanes 1 to 4 and lanes 5 to 8 represent experiments in which samples were electrophoresed for different lengths of time.

quired to inhibit 5S rRNA nuclear import is comparable to the concentration of WGA shown to inhibit the nuclear import of U6 small nuclear RNA, but 20 times less than the concentration required to inhibit nuclear import of U1 small nuclear RNA to a similar degree (see Fig. 3 in reference 48). These results correlate with studies on nuclear export of 5S rRNA and 5S rRNA-containing RNPs. Approximately 60% inhibition of ribosomal subunit export can be achieved when WGA is injected into the nucleus at a final concentration of 0.5 mg/ml (6), and export of 5S rRNA newly synthesized from microinjected cloned genes is significantly inhibited by preinjection of RL1, an antinucleoporin monoclonal antibody (24). The sensitivity of 5S rRNA to general inhibitors of nuclear transport suggests that 5S rRNA is targeted to oocyte nuclei by a receptor-mediated process.

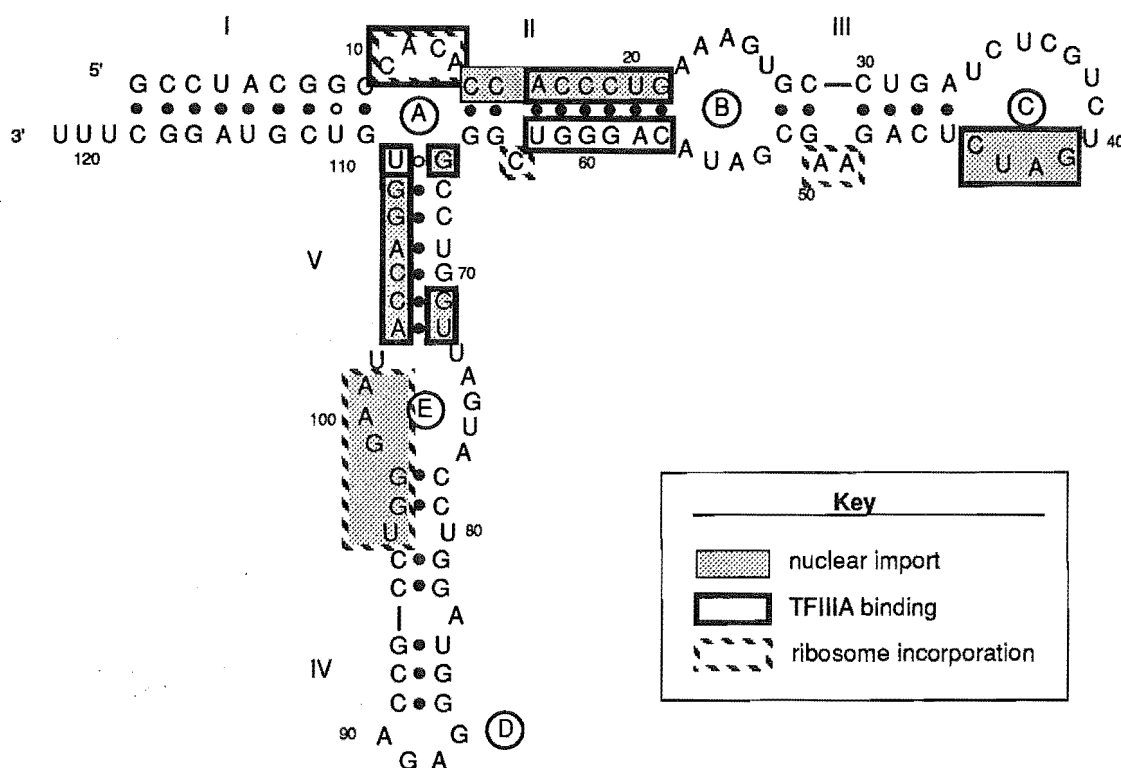


FIG. 9. Complex, nonidentical regions of 5S rRNA required for different functions. Shaded boxes indicate regions in which nucleotide substitution results in at least a 50% reduction in nuclear transport of 5S rRNA. Boxes outlined in bold indicate regions in which nucleotide substitution results in at least a 50% reduction in TFIIIA binding affinity. Boxes outlined in dashed lines indicate regions in which nucleotide substitution abolishes incorporation of 5S rRNA into the 60S ribosomal subunit.

Given the specificity of receptor-mediated processes, it seemed surprising that all mutants were capable of at least some degree of nuclear import. However, the results presented here are similar to an analysis of the nuclear export phenotypes of 30 different point mutants of human tRNA^{Met} which revealed variable defects in transport; the percentage of microinjected tRNA exported to the cytoplasm ranged from 85 (wild type) to 34% (D stem mutant) (67). Glyceraldehyde-3-phosphate dehydrogenase binds two defective mutants with lower affinity than that of wild-type tRNA, suggesting that this protein may be involved in tRNA export (62). This comparison with tRNA export and a model for the tertiary structure of 5S rRNA provide some insight into 5S rRNA import. 5S rRNA has been proposed to adopt a Y-shaped structure in which the three helical domains are independent (13, 71), that is, the effect that a mutation has on the RNA structure is primarily restricted to the mutated loop (12, 40). If a protein involved in nuclear targeting makes numerous contacts with 5S rRNA, disruption of one small region may not disrupt binding in other regions, therefore potentially providing a stable complex that is still recognized by the transport machinery. Relatively small changes in complex stability may be masked at the saturating levels of RNA used in these experiments.

Guddat et al. (31) proposed that 5S rRNA nuclear export is mediated by either TFIIIA or L5 on the basis of studies showing that mutant RNA molecules that do not form immunodetectable complexes with these proteins are retained in the nucleus. Results of this present study suggest that TFIIIA binding is not a prerequisite for nuclear import

of 5S rRNA, consistent with earlier work suggesting that 7S RNP transport is unidirectional (3, 44). A small nuclear pool of 7S RNPs containing labelled 5S rRNA was detected after microinjection of labelled 5S rRNA into the oocyte cytoplasm, but it is likely that these 7S RNPs were assembled after nuclear entry. If 5S rRNA enters the nucleus in excess of the amount required for assembly into nascent 60S ribosomal subunits, it may become associated with TFIIIA. Along these lines, it has been proposed that excess 5S rRNA may be targeted to the cytoplasm of mammalian somatic cells for degradation bound to a TFIIIA-like protein (39).

Ribosomal protein L5 and 5S rRNA form a stable complex prior to assembly of ribosomal subunits (3, 11, 64, 73). Cytoplasmic exchange between TFIIIA and L5 for binding of 5S rRNA correlates with the mobilization of 5S rRNA from storage within the *Xenopus* oocyte cytoplasm (3), suggesting that L5 may mediate 5S rRNA nuclear targeting. Little is known about the sequence and structural requirements for L5 binding to 5S rRNA, although chemical protection assays (35) and immunoprecipitation assays (3) indicate a binding domain similar to that of TFIIIA. Delineation of the sequence and structural requirements of 5S rRNA for L5 binding and correlation with data on subcellular localization should provide further insight into the role of L5 in nuclear import and nucleolar targeting of 5S rRNA. The possibility remains that other proteins, such as carriers that shuttle between the cytoplasm and the nucleus (9, 36, 46, 60), are involved in the subcellular trafficking of 5S rRNA.

5S rRNA structural elements required for ribosome assembly. Loop structures, bulged nucleotides, and non-Watson-

Crick base pairs of RNA have been shown to be of importance for specific protein recognition (4, 14, 54). Similarly, results presented here show that incorporation of 5S rRNA into ribosomes is abolished by deletion of bulged nucleotides, A at nucleotides 49 and 50 or C at nucleotide 63, and by nucleotide substitutions that alter the sequence of loop A or the noncanonical base pairing in loop E. These results correlate with results from other workers investigating prokaryotic 5S rRNA-protein interactions. *Escherichia coli* 5S rRNA has a structure similar to that of eukaryotic 5S rRNA, but *E. coli* 5S rRNA binds to three ribosomal proteins, L5, L18, and L25, rather than to one protein as in eukaryotes. A bulged nucleotide at position 65 in *E. coli* 5S rRNA, corresponding to position 63 in eukaryotic 5S rRNA, is necessary for binding of L18 to the 5S rRNA (reviewed in reference 29).

Various regions of 5S rRNA have been postulated to be of importance for ribosome assembly and function (69; for a review, see reference 29). Complementary base pairing between nucleotide sequences at the 5' and 3' ends of mouse 18S rRNA contained in the 40S ribosomal subunit and nucleotides 9 to 26 and 90 to 107 of 5S rRNA occurs in vitro (59). The functional significance of this stable interaction has yet to be determined. Interestingly, mutant 96-101 which has increased Watson-Crick base pairing in the loop E-helix IV region of 5S rRNA and mutant 10-13 which has an altered sequence in loop A were shown here to be defective in ribosome incorporation. These results indicate a correlation between the structural elements of the 5S rRNA molecule required for 60S ribosomal subunit assembly and the structural elements previously proposed to aid in formation of the 80S ribosome by 5S rRNA-nucleic acid interactions. Protein recognition of the noncanonical base pairing in loop E encompassing nucleotides 96 to 101 of the 5S rRNA molecule (57, 72) may be of importance for 60S ribosomal subunit assembly, as well as RNA-RNA hybridization.

5S rRNA represents an important model system for study of the regulated subcellular trafficking of RNA, because it involves many different components: shuttling of the 5S rRNA molecule across the nuclear envelope, a variety of RNA-protein interactions and exchanges, cytoplasmic localization of the 5S rRNA in RNPs (storage particles), and nucleolar targeting. Continued study of the effects of mutations by an in vitro binding assay (7, 8, 56, 74) and by the in vivo functional assays described here should help to further identify and clarify those sequence and structural elements of 5S rRNA required for some of its biological activities.

ACKNOWLEDGMENTS

This research was supported in part by grants from the University Grants Committee, Lottery Grants Board, and Health Research Council of New Zealand (to L.A.A.) and from the Natural Sciences and Engineering Research Council of Canada (NSERC) (to P.J.R.). P.J.R. is the recipient of an NSERC University Research Fellowship.

We are grateful to Renny Bishop for maintaining the *Xenopus* colony. We thank Alain Viel (Harvard University) and Adrienne Gomez de Gracia (Centre de Génétique Moléculaire) for help in preparation of the antisera used in this study. Thanks also are due to Dave Goldfarb and Neil Michaud (University of Rochester) for critical discussions of preliminary results and to Aimée Bakken (University of Washington) for helpful discussions.

REFERENCES

- Adam, S. A., and L. Gerace. 1991. Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import. *Cell* 66:837-847.
- Akey, C. W., and D. S. Goldfarb. 1989. Protein import through the nuclear pore complex is a multistep process. *J. Cell Biol.* 109:971-982.
- Allison, L. A., P. J. Romaniuk, and A. H. Bakken. 1991. RNA-protein interactions of stored 5S RNA with TFIIIA and ribosomal protein L5 during *Xenopus* oogenesis. *Dev. Biol.* 144:129-144.
- Bartel, D. P., M. L. Zapp, M. R. Green, and J. W. Szostak. 1991. HIV-1 Rev regulation involves recognition of non-Watson-Crick base pairs in viral RNA. *Cell* 67:529-536.
- Baserga, S. J., M. Gilmore-Hebert, and X. W. Yang. 1992. Distinct molecular signals for nuclear import of the nucleolar snRNA, U3. *Genes Dev.* 6:1120-1130.
- Bataillé, N., T. Helser, and H. M. Fried. 1990. Cytoplasmic transport of ribosomal subunits microinjected into the *Xenopus laevis* oocyte nucleus: a generalized, facilitated process. *J. Cell Biol.* 111:1571-1582.
- Baudin, F., and P. J. Romaniuk. 1989. A difference in the importance of bulged nucleotides and their parent base pairs in the binding of transcription factor IIIA to *Xenopus* 5S RNA and 5S RNA genes. *Nucleic Acids Res.* 17:2043-2056.
- Baudin, F., P. J. Romaniuk, P. Romby, C. Brunel, E. Westhof, B. Ehresmann, and C. Ehresmann. 1991. Involvement of "hinge" nucleotides of *Xenopus laevis* 5S rRNA in the RNA structural organization and in the binding of transcription factor TFIIIA. *J. Mol. Biol.* 218:69-81.
- Borer, R. A., C. F. Lehner, H. M. Eppenberger, and E. A. Nigg. 1989. Major nucleolar proteins shuttle between nucleus and cytoplasm. *Cell* 56:379-390.
- Breeuwer, M., and D. S. Goldfarb. 1990. Facilitated nuclear transport of histone H1 and other small nucleophilic proteins. *Cell* 60:999-1008.
- Brow, D. A., and E. P. Geiduschek. 1987. Modulation of yeast 5S RNA synthesis in vitro by ribosomal protein YL3: a possible regulatory loop. *J. Biol. Chem.* 262:13953-13958.
- Brunel, C., P. Romby, E. Westhof, P. J. Romaniuk, B. Ehresmann, and C. Ehresmann. 1990. Effect of mutations in domain 2 on the structural organization of oocyte 5S rRNA from *Xenopus laevis*. *J. Mol. Biol.* 215:103-111.
- Chow, C. S., K. M. Hartmann, S. L. Rawlings, P. W. Huber, and J. K. Barton. 1992. Delineation of structural domains in eukaryotic 5S rRNA with a rhodium probe. *Biochemistry* 31:3534-3542.
- Cullen, B. R. 1990. The HIV-1 Tat protein: an RNA sequence-specific processivity factor? *Cell* 63:655-657.
- Dabaualle, M.-C., B. Schulz, U. Scheer, and R. Peters. 1988. Inhibition of nuclear accumulation of karyophilic proteins in living cells by microinjection of the lectin wheat germ agglutinin. *Exp. Cell Res.* 174:291-296.
- Dargemont, C., and L. C. Kühn. 1992. Export of mRNA from microinjected nuclei of *Xenopus laevis* oocytes. *J. Cell Biol.* 118:1-9.
- Denis, H., and M. le Maire. 1983. Thesaurisomes, a novel kind of nucleoprotein particle, p. 263-267. In D. B. Roodyn (ed.), *Subcellular biochemistry*. Plenum Publishing Corp., New York.
- De Robertis, E. M., S. Lienhard, and R. F. Parisot. 1982. Intracellular transport of microinjected 5S and small nuclear RNAs. *Nature (London)* 295:572-577.
- Draper, D. E. 1989. How do proteins recognize specific RNA sites? New clues from autogenously regulated ribosomal proteins. *Trends Biochem. Sci.* 14:335-338.
- Dworetzky, S. I., and C. M. Feldherr. 1988. Translocation of RNA-coated gold particles through the nuclear pores of oocytes. *J. Cell Biol.* 106:575-584.
- Dworetzky, S. I., R. E. Lanford, and C. M. Feldherr. 1988. The effects of variations in the number and sequence of targeting signals on nuclear uptake. *J. Cell Biol.* 107:1279-1287.
- Eckner, R., W. Ellmeier, and M. L. Birnstiel. 1991. Mature mRNA 3' end formation stimulates RNA export from the nucleus. *EMBO J.* 10:3513-3522.
- Fabijanski, S., and M. Pellegrini. 1981. Identification of proteins at the peptidyl-tRNA binding site of rat liver ribosomes. *Mol. Gen. Genet.* 184:551-556.

24. Featherstone, C., M. K. Darby, and L. Gerace. 1988. A monoclonal antibody against the nuclear pore complex inhibits nucleocytoplasmic transport of protein and RNA *in vivo*. *J. Cell Biol.* **107**:1289-1297.
25. Finlay, D. R., and D. J. Forbes. 1990. Reconstitution of biochemically altered nuclear pores: transport can be eliminated and restored. *Cell* **60**:17-29.
26. Finlay, D. R., E. Meier, P. Bradley, J. Horecka, and D. J. Forbes. 1991. A complex of nuclear pore proteins required for pore function. *J. Cell Biol.* **114**:169-183.
27. Fischer, U., E. Darzynkiewicz, S. M. Tahara, N. A. Dathan, R. Lührmann, and I. W. Mattaj. 1991. Diversity in the signals required for nuclear accumulation of U snRNPs and variety in the pathways of nuclear transport. *J. Cell Biol.* **113**:705-714.
28. Fischer, U., V. Sumpter, M. Sekine, T. Satoh, and R. Lührmann. 1993. Nucleocytoplasmic transport of U snRNPs: definition of a nuclear location signal in the Sm core domain that binds a transport receptor independently of the m³G cap. *EMBO J.* **12**:573-583.
29. Garrett, R. A., S. Douthwaite, and H. F. Noller. 1981. Structure and role of 5S RNA-protein complexes in protein biosynthesis. *Trends Biochem. Sci.* **6**:137-139.
30. Goldfarb, D., and N. Michaud. 1991. Pathways for the nuclear transport of proteins and RNAs. *Trends Cell Biol.* **1**:20-24.
31. Guddat, U., A. H. Bakken, and T. Pieler. 1990. Protein-mediated nuclear export of RNA: 5S rRNA containing small RNPs in *Xenopus* oocytes. *Cell* **60**:619-628.
32. Hamm, J., E. Darzynkiewicz, S. M. Tahara, and I. W. Mattaj. 1990. The trimethylguanosine cap structure of U1 snRNA is a component of a bipartite nuclear targeting signal. *Cell* **62**:569-577.
33. Hamm, J., and I. W. Mattaj. 1990. Monomethylated cap structures facilitate RNA export from the nucleus. *Cell* **63**:109-118.
34. Holmberg, L., Y. Melander, and O. Nygård. 1992. Ribosome-bound eukaryotic elongation factor 2 protects 5S rRNA from modification. *J. Biol. Chem.* **267**:21906-21910.
35. Huber, P. W., and I. G. Wool. 1986. Use of the cytotoxic nuclease α -sarcin to identify the binding site on eukaryotic 5S ribosomal ribonucleic acid for the ribosomal protein L5. *J. Biol. Chem.* **261**:3002-3005.
36. Imamoto, N., Y. Matsuoaka, T. Kurihara, K. Kohno, M. Miyagi, F. Sakiyama, Y. Okada, S. Tsunasawa, and Y. Yoneda. 1992. Antibodies against 70-kD heat shock cognate protein inhibit mediated nuclear import of karyophilic proteins. *J. Cell Biol.* **119**:1047-1061.
37. Kelly, J. M., and R. A. Cox. 1982. The nucleotide sequence at the 3'-end of *Neurospora crassa* 18S-rRNA and studies on the interaction with 5S-rRNA. *Nucleic Acids Res.* **10**:6733-6745.
38. Khanna-Gupta, A., and V. C. Ware. 1989. Nucleocytoplasmic transport of ribosomes in a eukaryotic system: is there a facilitated transport process? *Proc. Natl. Acad. Sci. USA* **86**:1791-1795.
39. Lagaye, S., J.-P. Barque, M. le Maire, H. Denis, and C.-J. Larsen. 1988. Characterization by human antibodies of two HeLa cell proteins which are related to *Xenopus laevis* transcription factor TFIIIA. *Nucleic Acids Res.* **16**:2473-2487.
40. Leal de Stevenson, I., P. Romby, F. Baudin, C. Brunel, E. Westhof, C. Ehresmann, B. Ehresmann, and P. J. Romaniuk. 1991. Structural studies on site-directed mutants of domain 3 of *Xenopus laevis* oocyte 5S ribosomal RNA. *J. Mol. Biol.* **219**:243-255.
41. le Maire, M., and H. Denis. 1987. Biochemical research on oogenesis. Binding of tRNA to the nucleoprotein particles of *Xenopus laevis* previtellogenic oocytes. *J. Biol. Chem.* **262**:654-659.
42. Lo, A. C., and R. N. Nazar. 1982. Topography of 5.8S rRNA in rat liver ribosomes. *J. Biol. Chem.* **257**:3516-3524.
43. Martin, K., and A. Helenius. 1991. Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. *Cell* **67**:117-130.
44. Mattaj, I. W., S. Lienhard, R. Zeller, and E. M. DeRobertis. 1983. Nuclear exclusion of transcription factor IIIA and the 42S particle transfer RNA-binding protein in *Xenopus* oocytes: a possible mechanism for gene control? *J. Cell Biol.* **97**:1261-1265.
45. Mehlin, H., B. Daneholt, and U. Skoglund. 1992. Translocation of a specific pre-messenger ribonucleoprotein particle through the nuclear pore studied with electron microscope tomography. *Cell* **69**:605-613.
46. Meier, U. T., and G. Blobel. 1992. Nopp140 shuttles on tracks between nucleolus and cytoplasm. *Cell* **70**:127-138.
47. Michaud, N., and D. S. Goldfarb. 1991. Multiple pathways in nuclear transport: the import of U2 snRNP occurs by a novel kinetic pathway. *J. Cell Biol.* **112**:215-224.
48. Michaud, N., and D. Goldfarb. 1992. Microinjected U snRNAs are imported to oocyte nuclei via the nuclear pore complex by three distinguishable targeting pathways. *J. Cell Biol.* **116**:851-861.
49. Moore, M. S., and G. Blobel. 1992. The two steps of nuclear import, targeting to the nuclear envelope and translocation through the nuclear pore, require different cytosolic factors. *Cell* **69**:939-950.
50. Neuman de Vegvar, H. E., and J. D. Dahlberg. 1990. Nucleocytoplasmic transport and processing of small nuclear RNA precursors. *Mol. Cell. Biol.* **10**:3365-3375.
51. Newmeyer, D. D., and D. J. Forbes. 1988. Nuclear import can be separated into distinct steps *in vitro*: nuclear pore binding and translocation. *Cell* **52**:641-653.
52. Peters, R. 1984. Nucleo-cytoplasmic flux and intracellular mobility in single hepatocytes measured by fluorescence microphotolysis. *EMBO J.* **3**:1831-1836.
53. Pierandrei-Amaldi, P., and E. Beccari. 1980. Messenger RNA for ribosomal proteins in *Xenopus laevis* oocytes. *Eur. J. Biochem.* **106**:603-611.
54. Pruijn, G. J. M., R. L. Slobbe, and W. J. van Venrooij. 1991. Analysis of protein-RNA interactions within Ro ribonucleoprotein complexes. *Nucleic Acids Res.* **19**:5173-5180.
55. Richardson, W. D., A. D. Mills, S. M. Dilworth, R. A. Laskey, and C. Dingwall. 1988. Nuclear protein migration involves two steps: rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. *Cell* **52**:655-664.
56. Romaniuk, P. J. 1989. The role of highly conserved single-stranded nucleotides of *Xenopus* 5S RNA in the binding of transcription factor IIIA. *Biochemistry* **28**:1388-1395.
57. Romaniuk, P. J., I. Leal de Stevenson, C. Ehresmann, P. Romby, and B. Ehresmann. 1988. A comparison of the solution structures and conformational properties of the somatic and oocyte 5S rRNAs of *Xenopus laevis*. *Nucleic Acids Res.* **16**:2295-2312.
58. Romaniuk, P. J., I. Leal de Stevenson, and H.-H. A. Wong. 1987. Defining the binding site of *Xenopus* transcription factor IIIA on 5S RNA using truncated and chimeric 5S RNA molecules. *Nucleic Acids Res.* **15**:2737-2755.
59. Sarge, K. D., and E. S. Maxwell. 1991. Intermolecular hybridization of 5S rRNA with 18S rRNA: identification of a 5'-terminally-located nucleotide sequence in mouse 5S rRNA which base-pairs with two specific complementary sequences in 18S rRNA. *Biochim. Biophys. Acta* **1088**:57-70.
60. Shi, Y., and J. O. Thomas. 1992. The transport of proteins into the nucleus requires the 70-kilodalton heat shock protein or its cytosolic cognate. *Mol. Cell. Biol.* **12**:2186-2192.
61. Silver, P. 1991. How proteins enter the nucleus. *Cell* **64**:489-497.
62. Singh, R., and M. R. Green. 1993. Sequence-specific binding of transfer RNA by glyceraldehyde-3-phosphate dehydrogenase. *Science* **259**:365-368.
63. Stebbins-Boaz, B., and S. A. Gerbi. 1991. Structural analysis of the peptidyl transferase region in ribosomal RNA of the eukaryote *Xenopus laevis*. *J. Mol. Biol.* **217**:93-112.
64. Steitz, J. A., C. Berg, J. P. Hendrick, H. La Branche-Chabot, A. Metspalu, J. Rinke, and T. Yario. 1988. A 5S rRNA/L5 complex is a precursor to ribosome assembly in mammalian cells. *J. Cell Biol.* **106**:545-556.
65. Sterne-Marr, R., J. M. Blevitt, and L. Gerace. 1992. O-linked glycoproteins of the nuclear pore complex interact with a cytosolic factor required for nuclear protein import. *J. Cell Biol.* **116**:271-280.

66. Theunissen, O., F. Rudt, U. Guddat, H. Mentzel, and T. Pieler. 1992. RNA and DNA binding zinc fingers in *Xenopus* TFIIIA. *Cell* 71:679-690.
67. Tobian, J. A., L. Drinkard, and M. Zasloff. 1985. tRNA nuclear transport: defining the critical regions of human tRNA^{met} by point mutagenesis. *Cell* 43:415-422.
68. Uchiumi, T., M. Kikuchi, and K. Ogata. 1986. Cross-linking study on protein neighborhoods at the subunit interface of rat liver ribosomes with 2-iminothiolane. *J. Biol. Chem.* 261:9663-9667.
69. Van Ryk, D. I., Y. Lee, and R. N. Nazar. 1992. Unbalanced ribosome assembly in *Saccharomyces cerevisiae* expressing mutant 5S rRNAs. *J. Biol. Chem.* 267:16177-16181.
70. Viel, A., M.-J. Armand, J.-C. Callen, A. Gomez de Gracia, H. Denis, and M. le Maire. 1990. Elongation factor 1 α (EF-1 α) is concentrated in the balbiani body and accumulates coordinately with the ribosomes during oogenesis of *Xenopus laevis*. *Dev. Biol.* 141:270-278.
71. Westhof, E., P. Romby, P. J. Romaniuk, J.-P. Ebel, C. Ehresmann, and B. Ehresmann. 1989. Computer modeling from solution data of spinach chloroplast and of *Xenopus laevis* somatic and oocyte 5S rRNAs. *J. Mol. Biol.* 207:417-431.
72. Wimberly, B., G. Varani, and I. Tinoco, Jr. 1993. The conformation of loop E of eukaryotic 5S ribosomal RNA. *Biochemistry* 32:1078-1087.
73. Wormington, W. M. 1989. Developmental expression and 5S rRNA-binding activity of *Xenopus laevis* ribosomal protein L5. *Mol. Cell. Biol.* 9:5281-5288.
74. You, Q., and P. J. Romaniuk. 1990. The effects of disrupting 5S RNA helical structures on the binding of *Xenopus* transcription factor IIIA. *Nucleic Acids Res.* 18:5055-5062.
75. Zasloff, M. 1983. tRNA transport from the nucleus in a eukaryotic cell: carrier-mediated translocation process. *Proc. Natl. Acad. Sci. USA* 80:6436-6440.

II. The attached reprint from *Developmental Biology* contains some of the research I performed during my PhD studies. These results were not presented in this thesis. My contribution to this publication consisted of the analyses of oocyte-type and somatic-type 5S RNA ribonucleoprotein particle formation throughout oocyte development.

Signature

 31-1-96

Differential Binding of Oocyte-Type and Somatic-Type 5S rRNA to TFI_{II}A and Ribosomal Protein L5 in *Xenopus* Oocytes: Specialization for Storage versus Mobilization

LIZABETH A. ALLISON,¹ MELANIE T. NORTH, AND LOUISE A. NEVILLE

Department of Zoology, University of Canterbury, Christchurch, New Zealand

Accepted December 7, 1994

We studied the pathway of 5S ribosomal RNA (rRNA) during oogenesis in *Xenopus* from its storage in the cytoplasm to incorporation into ribosomes in the nucleus. Ribonucleoprotein particle (RNP) assembly assays reveal striking differences in the behavior of oocyte-type and somatic-type 5S rRNA after microinjection into stage II, III, or IV oocytes or into the cytoplasm of stage V–VI oocytes. Microinjected oocyte-type 5S rRNA predominantly interacts with the 5S rRNA gene-specific transcription factor IIIA (TFI_{II}A) to form storage 7S RNPs. In contrast, microinjected somatic-type 5S rRNA predominantly interacts with ribosomal protein L5 to form 5S RNPs, which are precursors to ribosome assembly. In addition, a greater amount of somatic-type 5S rRNA accumulates in the nucleus and is assembled into 60S ribosomal subunits. Thus, a slight difference in nucleotide sequence results in differential binding of 5S rRNA to TFI_{II}A and L5, specializing oocyte-type for storage in the oocyte cytoplasm and somatic-type for rapid mobilization and ribosome assembly. When oocyte-type and somatic-type 5S rRNA molecules were microinjected into the nucleus of stage V–VI oocytes in excess of other ribosomal components, the nucleocytoplasmic distribution of both types of RNA was similar, but the distinctive protein associations were maintained. In contrast, the behavior of oocyte-type and somatic-type 5S rRNA gradually synthesized *in situ* from microinjected cloned genes was similar, suggesting that nascent RNA is rapidly and directly recruited into ribosomes, thus bypassing an excursion into the cytoplasm prior to ribosome assembly. © 1995

Academic Press, Inc.

INTRODUCTION

Xenopus has two families of 5S ribosomal RNA (rRNA) genes, oocyte-type and somatic-type, which are under developmental control and produce 5S rRNAs

differing in six nucleotides (Wegnez *et al.*, 1972; Ford and Southern, 1973; reviewed by Wolffe and Brown, 1988). The oocyte-type family of 5S rRNA genes, with approximately 20,000 copies per haploid genome, is actively transcribed in the developing oocyte yielding large amounts of 5S rRNA for ribosome stockpiling, but is relatively inactive during early embryogenesis and is switched off in somatic cells. In contrast, the somatic-type family of 5S rRNA genes, with approximately 400 copies per haploid genome, is transcribed throughout development, including in oocytes (Wormington and Brown, 1983). There has been widespread interest in the 5S rRNA gene system as a model for differential gene regulation (e.g., Wormington *et al.*, 1981; McConkey and Bogenhagen, 1988; Keller *et al.*, 1990; reviewed by Wolffe and Brown, 1988), but less attention has been devoted to defining functional differences in the corresponding RNAs.

Preribosomal particles containing 5S, 18S, 5.8S, and 28S rRNAs, and many of the ribosomal proteins, are assembled in the nucleoli of somatic cells and are exported to the cytoplasm as 40S and 60S ribosomal subunits (reviewed by Hadjiolov, 1985). In previtellogenic (stages I to II; Dumont, 1972) *Xenopus* oocytes, however, 5S rRNA is synthesized before other components of ribosomes are available (Mairy and Denis, 1971) and stored in the cytoplasm as 7S ribonucleoprotein particles (RNPs) complexed with the 5S rRNA gene-specific transcription factor IIIA (TFI_{II}A) (Picard and Wegnez, 1979; Honda and Roeder, 1980; Pelham and Brown, 1980) or with other nonribosomal proteins and tRNA as 42S RNPs (Picard *et al.*, 1980). Later in oogenesis (stages III to VI), when the synthesis of other ribosomal constituents is maximal, 5S rRNA becomes associated with ribosomal protein L5, forming 5S RNPs, and returns to the nucleus for incorporation into ribosomes in the amplified nucleoli (Allison *et al.*, 1991, 1993). At this stage, newly synthesized 5S rRNA may follow a shorter pathway for ribosome assembly. Nascent 5S rRNA first forms a transient association with the La protein (Guddat *et al.*, 1990),

¹ To whom correspondence should be addressed at the Department of Zoology, University of Canterbury, Private Bag 4800, Christchurch, New Zealand. Fax: (64) (3) 364-2024. e-mail: l.allison@csc.canterbury.ac.nz.

which may be involved in transcription termination (Gottlieb and Steitz, 1989). After La is replaced by L5, 5S rRNA may then migrate from the site of transcription directly to the nucleoli for incorporation into ribosomes, as is proposed for somatic cells (Steitz *et al.*, 1988).

Since there are 50-fold more of the oocyte-type genes, oocyte-type predominates as the major form of 5S rRNA in oocytes. Somatic-type 5S rRNA synthesized in oocytes has been shown to be incorporated into RNPs immediately after synthesis; however, only storage particles containing oocyte-type 5S rRNA appear to be conserved over time. Somatic-type 5S rRNA has not been detected in the pool of RNPs or ribosomes stored for many months in the oocyte for use during embryogenesis (Denis and Wegnez, 1977). In this investigation, we asked whether the six nucleotide substitutions distinguishing the two types of 120 nucleotide RNA molecule make oocyte-type 5S rRNA better suited than somatic-type 5S rRNA to the unusual way in which ribosomes are assembled during oogenesis. We tested whether somatic-type 5S rRNA is capable of following individual steps in the oocyte-type pathway of storage in the cytoplasm and subsequent return to the nucleus for assembly into ribosomal subunits.

First, in order to mimic the pathway followed by stored 5S rRNA during oogenesis, we microinjected *in vitro*-generated oocyte-type and somatic-type 5S rRNA into stage II, III, or IV oocytes or into the cytoplasm of stage V–VI oocytes. The results of RNP assembly assays reported here reveal striking differences in the behavior of the two types of RNA. Oocyte-type 5S rRNA predominantly interacts with TFIIA to form storage 7S RNPs, while somatic-type 5S rRNA predominantly interacts with L5 to form 5S RNPs, which are precursors to ribosome assembly. In addition, we show that twice as much somatic-type 5S rRNA accumulates in the nucleus and a greater amount is assembled into nascent 60S ribosomal subunits. Thus, the nucleotide substitutions distinguishing the two types of 5S rRNA result in differential binding to TFIIA and L5 *in vivo*, specializing oocyte-type for long-term storage in the oocyte cytoplasm and somatic-type for rapid mobilization and ribosome assembly. Next, in order to track 5S rRNA originating in the nucleus in excess of other ribosomal components, we microinjected labeled oocyte-type and somatic-type 5S rRNA into the nucleus of stage V–VI oocytes. Under these conditions, the nucleocytoplasmic distribution of oocyte-type and somatic-type 5S rRNA was similar, but the distinctive protein associations were maintained. Finally, we compared injection of 5S rRNA into the nucleus *en masse* with gradual synthesis *in situ*. When we injected cloned genes encoding the two types of RNA into oocyte nuclei, we found that the behavior of newly transcribed oocyte-type and somatic-type 5S rRNA was similar. This distribution most likely reflects a more

rapid and direct recruitment of newly synthesized 5S rRNA into nascent ribosomes, thus bypassing an excursion into the cytoplasm prior to ribosome assembly.

MATERIALS AND METHODS

Plasmids and Synthesis of 5S rRNA

Internally labeled 5S rRNAs were produced by *in vitro* transcription from pXlo-wt (oocyte-type) and pXls-wt (somatic-type) gene templates (generous gifts from P. J. Romaniuk, University of Victoria, Victoria, British Columbia, Canada). These 5S rRNA genes were constructed so that the T7 transcripts have 5' and 3' termini identical to those found in the natural 5S rRNA molecules (Romaniuk *et al.*, 1987). Transcription reactions were performed using 50 U T7 RNA polymerase (Epicentre Technologies: Intermed Scientific Ltd., Auckland, New Zealand) and 50–100 μ Ci [α - 32 P]GTP (3000 Ci/mmol; Amersham Australia Pty. Ltd. or DuPont NEN: Life Technologies Ltd., Auckland, New Zealand) in a 20- μ l reaction containing transcription buffer (Epicentre), 10 mM dithiothreitol, 40 U RNasin (Promega: Pacific Diagnostics Pty. Ltd., Auckland, New Zealand), 200 μ M CTP, ATP, and UTP, 40 μ M GTP, and 1 μ g DNA template linearized with *Dra*I (Boehringer Mannheim N. Z. Ltd., Auckland, New Zealand). The mixture was incubated for 1 hr at 37°C followed by treatment with 1 U RNase-free DNase (Boehringer Mannheim). Subsequently, 130 μ l UB Blue (175 mM NaCl, 5 mM Tris, pH 7.4, 5 mM EDTA, 0.5% SDS, 0.05% methylene blue, 7 M urea) and 20 μ g of glycogen (Boehringer Mannheim) were added. The sample was phenol/chloroform extracted and the RNA transcripts were precipitated 15 min on ice with 2 vol ethanol. A second precipitation was carried out with 2.5 M ammonium acetate and 2 vol ethanol. The RNA pellet was resuspended in TE (10 mM Tris-HCl, pH 7.6, 1 mM EDTA) at 100,000 cpm/ μ l and stored at –80°C. RNA concentration was quantified by DNA Quik STRIP (Eastman Chemical Co., New Haven, CT).

In one experiment, we tested whether there was a requirement for renaturation of *in vitro*-generated oocyte-type and somatic-type 5S rRNA prior to microinjection. RNA samples were resuspended in 20 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, and 50 mM KCl. The RNA was then renatured by heating to 65°C for 10 min, followed by slow (1 hr) cooling to room temperature. There was no difference in the behavior of microinjected renatured 5S rRNA, compared with 5S rRNA stored in TE; thus, this step was not carried out in subsequent experiments.

Plasmids encoding 5S rRNA genes for nuclear injections were obtained from A. H. Bakken (University of Washington, Seattle, WA): pXlo8 contains four *Xenopus laevis* oocyte-type 5S rRNA gene repeats (described in Birkenmeier *et al.*, 1978) and pJH17 contains one so-

matic-type 5S rRNA gene repeat (constructed by J. Hannas, University of Oklahoma Health Sciences Center).

Microinjection

Stage V–VI *X. laevis* oocytes were microinjected with 20 nl RNA (2000 cpm/oocyte) into the cytoplasm or nucleus using previously published procedures (Allison *et al.*, 1993). A range of concentrations were tested; injections of 0.2 to 2 ng per oocyte yielded the same nucleocytoplasmic and RNP distribution. Where results of different treatments were being compared, an identical amount of oocyte-type and somatic-type 5S rRNA was injected for each treatment. To control nuclear injection, the technique of Jarmolowski *et al.* (1994) was followed. Samples were mixed (1:1) with a 20-mg/ml solution of filter-sterilized blue dextran (2,000,000 molecular weight) (Sigma Chemical Co., St. Louis, MO). After dissection, only oocytes with blue nuclei were used. This procedure had no apparent effect on nuclear transport or RNP assembly. For analysis of newly synthesized RNA, 20 nl plasmid DNA (0.25 mg/ml) was microinjected into the nucleus together with [α - 32 P]GTP. The amount of RNA synthesized *in situ* over 20 hr was estimated to be approximately 0.1 to 0.2 ng, based on the amount of DNA injected and the rate of rRNA synthesis in oocytes (Gurdon and Melton, 1981).

For injections of immature oocytes, oocytes were first separated into Dumont stages (Dumont, 1972) using the following criteria: late stage II, white/opaque, 300 μ m; stage III, tan, 450 μ m; stage IV, dark brown, distinct animal hemisphere, 600 μ m. Staged oocytes were immobilized in culture dishes containing 0.7% agarose in O-R2 with depressions of the appropriate diameter and microinjected with 2 nl of *in vitro*-generated RNA.

Analysis of Nuclear Transport

After microinjection and incubation in O-R2 medium, nuclei were manually dissected from oocytes in 1% TCA and collected for analysis (Allison *et al.*, 1991). RNA was extracted from nuclear and cytoplasmic fractions according to a procedure modified from Xie and Rothblum (1991). Three cytoplasms or nuclei (plus two carrier uninjected oocytes) were homogenized on ice in 30 μ l O-R2 by vigorously pipetting with a Gilson P200 Pipetman. Nine hundred seventy microliters of solution A (TE-saturated phenol, solution B, and 2 M NaOAc, pH 4.0, in a ratio of 1:1:0.1, supplemented with 720 μ l β -mercaptoethanol per 100 ml of solution B) was immediately added to homogenates. Solution B consisted of 4 M guanidinium thiocyanate and 25 mM sodium citrate (pH 7.0). One hundred microliters of chloroform/isoamyl alcohol (24:1) was added to each tube and the phases were mixed by vortexing briefly. The mixture was kept on ice for 30 min and the phases were separated by centrifuging at 9000g

for 20 min. The upper aqueous phase was recovered and the RNA was precipitated at -20°C by addition of an equal volume of isopropanol. The RNA was analyzed by 8% polyacrylamide/8 M urea gel electrophoresis as described (Allison *et al.*, 1993). Dried gels were autoradiographed on Amersham Hyperfilm-MP at -80°C . A Zeineh analytical hand-held scanning densitometer and Biomed Image Analysis software (Advanced American Biotechnology, Fullerton, CA) were used to quantify the intensity of bands on suitable exposures of autoradiograms (within the linear range of signal intensity of the film). In addition, samples were quantified prior to electrophoresis in a Quick-Count QC-2000 benchtop radioisotope counter (Bioscan, Inc., Washington, DC).

Analysis of RNP Formation

Crude cellular lysates were prepared from samples of either three stage V–VI or five stage II, III, or IV microinjected oocytes as described in Allison *et al.* (1993). Samples were electrophoresed on 6% polyacrylamide/0.1% Triton X-100 gels in TBE with 0.1% Triton X-100, followed by autoradiography and densitometric analysis. A sample of unlabeled, native 7S RNPs (5S rRNA complexed with TFIIIA), purified from immature *Xenopus* ovaries as described in Allison *et al.* (1991), were included as a marker. The marker lane was stained with ethidium bromide and viewed by uv illumination. To prepare a marker for 5S rRNA complexed with ribosomal protein L5, native 5S RNPs were isolated from EDTA-treated ribosomes from *Xenopus* ovaries as described in Allison *et al.* (1991). An exchange reaction was then used to incorporate internally labeled 5S rRNA into the native RNPs, following the methods of Huber and Wool (1986). ^{32}P -labeled oocyte-type 5S rRNA was incubated at 0 – 4°C for 1 hr in 20 μ l of 25 mM EDTA, pH 7.0, containing approximately 5 μ g of 5S RNPs. The exchange reaction was stopped by the addition of MgCl_2 to a final concentration of 25 mM.

The protein components of labeled 5S rRNA-containing RNPs formed *in situ* were analyzed as follows. After incubation for 24 hr, five stage V oocytes microinjected with oocyte-type or somatic-type 5S rRNA were homogenized together with 60 uninjected stage III oocytes in order to supplement the pool of nonribosome bound 5S rRNA-containing RNPs. After separation of RNPs by nondenaturing polyacrylamide gel electrophoresis, the 0.75-mm gel was dried and an autoradiogram template was prepared. RNP bands were excised and gel slices were rehydrated with 30 μ l sterile ddH_2O , removed from the filter paper backing, and transferred to a 1.5-ml microcentrifuge tube on ice. Twenty microliters of sample buffer (2% SDS, 100 mM dithiothreitol, 60 mM Tris, pH 6.8, 0.01% bromophenol blue) was added and the gel slices were heated at 100°C for 5 min. Replicate gel

slices, permeated with sample buffer, were then transferred to the wells of a 1.0-mm-thick 12% polyacrylamide gel containing 0.1% SDS. After electrophoresis for 8 hr at 10 V/cm, half the gel was stained with 0.25% Coomassie blue R250 to visualize proteins. To visualize RNA and proteins, the other half of the gel was silver stained with a Bio-Rad silver stain kit (Bio-Rad Laboratories Pty. Ltd., Auckland, New Zealand) according to the manufacturer's instructions.

Antiserum and Immunoprecipitation Assays

Anti-60S ribosomal subunit antiserum raised against *Xenopus* 60S ribosomal subunits was a generous gift from M. le Maire (CEA et CNRS, Gif-sur-Yvette, France). The antiserum is described and characterized in Viel *et al.* (1990) and Allison *et al.* (1993).

Microinjected stage V–VI oocytes were incubated for 45 hr at 18°C. Nuclei were isolated in 25 mM Tris–HCl, pH 8.0, 10% glycerol, 5 mM MgCl₂, and 2 mM dithiothreitol (Allison *et al.*, 1991). Cleared homogenates of 20 nuclear and cytoplasmic fractions from microinjected oocytes were prepared as described in Allison *et al.* (1991), and immunoprecipitation assays were carried out using 20 μ l antiserum bound to protein A–Sepharose (Pharmacia LKB Biotechnology, Auckland, New Zealand). RNA was recovered from the immunoprecipitates and the immunosupernatants and resolved on 8% polyacrylamide/8 M urea gels followed by autoradiography. Samples were quantified as described above for nuclear transport assays.

For analysis of ribosome assembly during earlier stages of oogenesis, microinjected stage II, III, and IV oocytes were incubated for 24 hr at 18°C. Immunoprecipitation assays were then carried out on cleared homogenates of 20 oocytes.

RESULTS

The Nuclear Import Kinetics of Oocyte-Type and Somatic-Type 5S rRNA Are Different

Nuclear import of oocyte-type 5S rRNA occurs by a temperature- and energy-dependent, nuclear pore-mediated process (Allison *et al.*, 1993). Since the six somatic-specific nucleotide substitutions of 5S rRNA (Fig. 1) are located between nucleotides 11 and 108, in the region of the molecule shown to provide the structural information required for nuclear transport (Allison *et al.*, 1991, 1993), it was of interest to determine whether these substitutions alter the nuclear transport characteristics of 5S rRNA. To investigate nuclear import of the two types of RNA, we microinjected *in vitro*-generated oocyte-type and somatic-type 5S rRNA into the cytoplasm of stage V–VI oocytes, thus mimicking the pathway followed by stored 5S rRNA during oogenesis. A kinetic

analysis of nuclear import of the two types of RNA revealed that somatic-type 5S rRNA enters the nucleus more rapidly and accumulates to a greater extent than oocyte-type (Figs. 2A and 2B). At maximal levels, 35% of microinjected somatic-type 5S rRNA was found in the nucleus, while oocyte-type 5S rRNA reached levels of only 19%. This percentage of total RNA in the nucleus represents a state of flux between the cytoplasm and the nucleus; 5S rRNA migrates into the nucleus and then returns to the cytoplasm, presumably after assembly into ribosomal subunits (Allison *et al.*, 1991, 1993). Six additional experiments with oocytes from different frogs confirmed this pattern of distribution. On average, nuclear accumulation of somatic-type 5S rRNA after approximately 20 hr incubation was twice that of oocyte-type 5S rRNA (Table 1). The kinetics of oocyte-type 5S rRNA transport shown here are comparable to our previous reports (Allison *et al.*, 1991, 1993).

To compare the nucleocytoplasmic distribution of cytoplasmically injected 5S rRNA with 5S rRNA originating in the nucleus in excess of other ribosomal components, we next microinjected *in vitro*-generated 5S rRNA into oocyte nuclei. After 20 hr incubation both types of RNA were predominantly localized in the nucleus (Fig. 2B; Table 1). On average, 62% of oocyte-type and 80% of somatic-type 5S rRNA were found in the nucleus.

Finally, to compare the nuclear export characteristics of 5S rRNA injected *en masse* into the nucleus with RNA gradually synthesized *in situ*, we introduced radio-labeled 5S rRNA molecules by transcription from microinjected gene templates. After 3 hr incubation, slightly more newly synthesized somatic-type 5S rRNA was found in the cytoplasm, compared with oocyte-type 5S rRNA (Fig. 2B). However, after 20 hr incubation, the nucleocytoplasmic distribution of the two types of RNA was similar (Fig. 2B; Table 1). On average, 68% of oocyte-type and 60% of somatic-type 5S rRNA were found in the nucleus. During this incubation period a negligible amount of 5S rRNA was synthesized from endogenous genes (Fig. 2C).

In summary, we have shown that after microinjection into the oocyte cytoplasm, somatic-type 5S rRNA enters the nucleus more rapidly and accumulates to a greater extent than oocyte-type. These findings suggest that somatic-specific substitutions enhance nuclear import of 5S rRNA. In contrast, the nuclear export characteristics of the two types of 5S rRNA are comparable. After nuclear microinjection, a greater percentage of both types of RNA was found in the nucleus when compared with their distribution after microinjection into the cytoplasm. These results suggest that the efficiency of export was low, possibly due to saturation of a specific event in export. However, the concentration dependence of 5S rRNA export is not a simple function (Jarmolowski

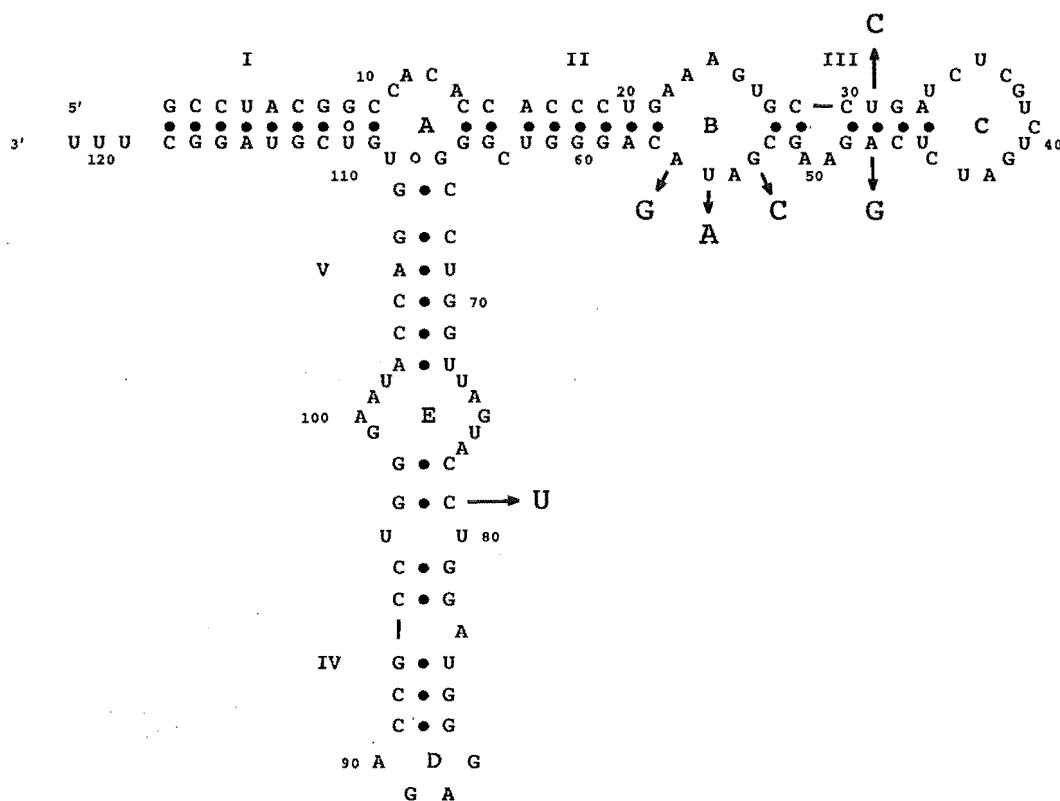


FIG. 1. Somatic-specific nucleotide substitutions of *Xenopus laevis* 5S rRNA. The secondary structure of *X. laevis* oocyte-type 5S rRNA is shown. Somatic-specific nucleotide substitutions are indicated at residue numbers 30, 47, 53, 55, 56, and 79.

et al., 1994), and the percentage of total RNA in the nucleus may also represent reimport of 5S rRNA.

Oocyte-Type and Somatic-Type 5S rRNA Bind Differentially to TFI_{II}A and L5

Since the above experiments revealed significant differences in the nuclear import characteristics of oocyte-type and somatic-type 5S rRNA, it was of interest to determine whether these differences could be correlated with the protein associations formed upon introduction of 5S rRNA into the oocyte cytoplasm or nucleus. Although 5S rRNA-protein interactions *in vivo* have been analyzed previously (Guddat *et al.*, 1990; Allison *et al.*, 1991, 1993), no systematic comparison has been made between the two types of RNA.

To determine whether there are differences in protein binding of oocyte-type and somatic-type 5S rRNA, quantitative analysis of RNP formation was performed directly by electrophoresis of oocyte homogenates on nondenaturing gels. Association of 5S rRNA with the 34-kDa protein L5 (Wormington, 1989) results in a 5S RNP complex of intermediate mobility, migrating between unbound 5S rRNA and RNA associated with the 38.5-kDa protein TFI_{II}A (Ginsberg *et al.*, 1984) (Fig. 3; cf. Fig. 2 in Sands and Bogenhagen, 1991). In an earlier

study, the identity of this intermediate band had not been confirmed (Allison *et al.*, 1993). We show here that this band migrates with the same mobility as native 5S RNPs, isolated from EDTA-treated ribosomes from *Xenopus* ovaries (Fig. 3B, lane 1). The identity of the protein component of these native, purified 5S RNPs has been confirmed by Western analysis using an antibody against L5 (data not shown). Bands from a gel similar to the one shown in Fig. 3B were excised and the components electroeluted directly into an SDS-polyacrylamide gel. The single protein present in the 5S RNP band migrates with an apparent molecular weight of 34 kDa (Fig. 3A, lane 3).

Figure 3B shows a kinetic analysis of the RNP distribution of oocyte-type and somatic-type 5S rRNA after microinjection into the cytoplasm of stage V-VI oocytes. Densitometric analysis of the final RNP distribution is presented in Table 1. Approximately 50% of protein-bound oocyte-type 5S rRNA first appears as an RNP band migrating more slowly than the 7S RNP and 5S RNP bands (Fig. 3B, lanes 2 and 3). This transient complex may represent association of 5S rRNA with p43, the 43-kDa 5S rRNA-binding component of 42S RNPs (reviewed by Denis and le Maire, 1983; Sands and Bogenhagen, 1991). The concentration of 42S RNPs drops sharply

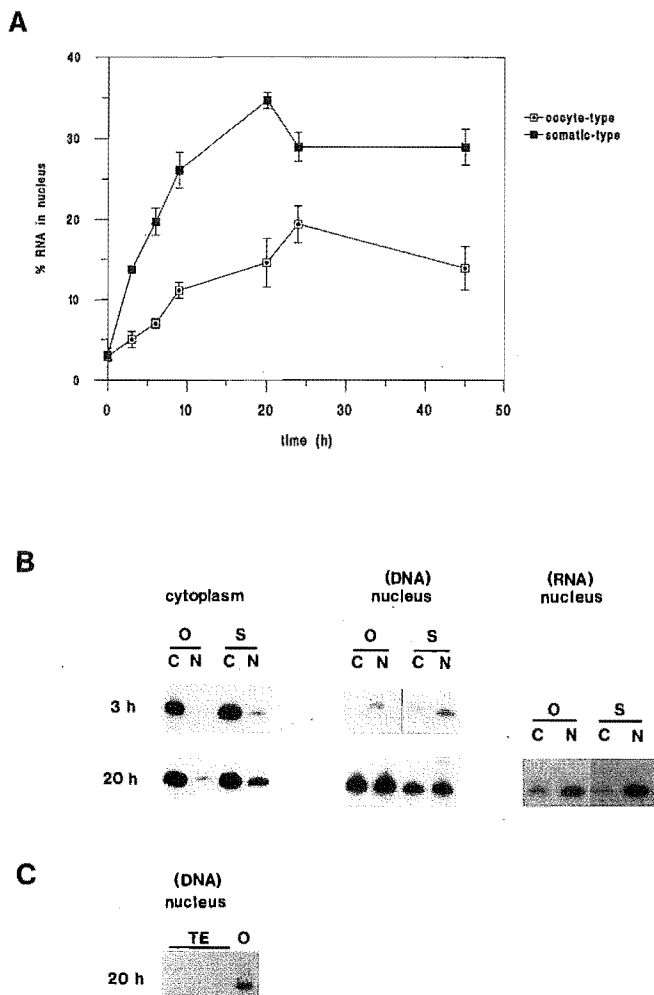


FIG. 2. Differential kinetics of somatic-type and oocyte-type 5S rRNA nuclear transport. (A) Quantitation of nuclear accumulation after microinjection into the cytoplasm. Oocytes were microinjected with ^{32}P -labeled oocyte-type or somatic-type 5S rRNA and incubated for the time intervals indicated. After manual dissection, RNA was extracted from three pooled nuclear and cytoplasmic fractions and subjected to denaturing electrophoresis. The percentage of radioactivity in the nucleus was quantified by densitometry on suitable exposures of autoradiograms (within the linear range of signal intensity of the film). Each point is the mean of two to three groups of three oocytes. The error bars indicate the standard error of means. (B) Comparison of the nucleocytoplasmic distribution of oocyte-type and somatic-type 5S rRNA after microinjection of labeled RNA into the cytoplasm (autoradiogram exposure time, 5 hr), [$\alpha\text{-}^{32}\text{P}$]GTP and cloned genes (DNA) into the oocyte nucleus (exposure time, 25 hr), or labeled RNA into the nucleus (exposure time, 2 hr). RNA was isolated from both nuclear (N) and cytoplasmic (C) fractions after 3 or 20 hr incubation as indicated and analyzed by denaturing electrophoresis. O, oocyte-type 5S rRNA. S, somatic-type 5S rRNA. (C) Analysis of the relative amount of 5S rRNA synthesized from endogenous genes during a 20-hr incubation. [$\alpha\text{-}^{32}\text{P}$]GTP and either an oocyte-type 5S rRNA gene template (O) or buffer (TE) were microinjected into the oocyte nucleus.

at the onset of vitellogenesis (Dixon and Ford, 1982; Viel *et al.*, 1990); however, after microinjection into stage V–VI oocytes detectable amounts of labeled 5S rRNA are

recovered by immunoprecipitation with anti-42S RNP antibodies (data not shown). Analysis of the proteins from this RNP band revealed a predominant protein migrating with an apparent molecular weight of 43 kDa along with other minor species (Fig. 3A, lane 5). Twenty percent of somatic-type 5S rRNA was also present in this higher molecular weight complex, but approximately 45% of somatic-type immediately associated with L5 upon introduction into the oocyte cytoplasm (Fig. 3B, lanes 4 and 5).

With increasing incubation time, the two types of 5S rRNA formed stable complexes with either TFIIA or L5 in a strikingly different pattern (Fig. 3B, cf. lanes 27 and 28). Microinjected somatic-type 5S rRNA predominantly interacts with L5 to form 5S RNPs, which are precursors to ribosome assembly. In contrast, microinjected oocyte-type 5S rRNA predominantly interacts with TFIIA to form storage 7S RNPs. Analysis of the relative RNP distribution in five batches of oocytes from different frogs revealed the same distinctive pattern, although the absolute amounts of protein-bound 5S rRNA were variable (Table 1). Injecting one-tenth the amount of RNA also resulted in the same pattern of distribution (data not shown). Thus, there is a correlation between the predominant association of somatic-type 5S rRNA with L5 and its entering the nucleus more rapidly and accumulating to a greater extent than oocyte-type 5S rRNA. These findings suggest that the somatic-specific substitutions enhance nuclear import of 5S rRNA by increasing the affinity of the RNA molecule for L5, rather than by their direct interaction with other components of the nuclear transport machinery.

To determine whether this distinctive pattern of RNP distribution was exhibited by 5S rRNA originating in the nucleus in excess of other ribosomal components, *in vitro*-generated 5S rRNA was microinjected into stage V–VI oocyte nuclei. A similar pattern was observed for somatic-type 5S rRNA, with the majority of the RNA associating with L5 to form preribosomal 5S RNPs (Fig. 3C, lane 3; Table 1). Oocyte-type 5S rRNA showed little association with L5, but also a decreased association with TFIIA (Fig. 3C, lane 1; Table 1), most likely reflecting a smaller pool of TFIIA in the nucleus than in the cytoplasm of stage V–VI oocytes.

Finally, we investigated whether this distinctive pattern of RNP distribution after nuclear injection *en masse* was exhibited by 5S rRNA gradually synthesized *in situ*. After microinjection of cloned genes into oocyte nuclei, a different pattern was revealed. Both types of newly synthesized RNA were associated to a greater extent with L5 than with TFIIA (Fig. 3C, lanes 2 and 4; Table 1).

Differential RNP Distribution throughout Oogenesis

To investigate whether the differential RNP distribution observed in stage V–VI oocytes is solely a feature

TABLE 1
SUMMARY OF THE DIFFERENTIAL NUCLEAR TRANSPORT AND RNP FORMATION OF OOCYTE-TYPE
AND SOMATIC-TYPE 5S rRNA IN STAGE V-VI *Xenopus* OOCYTES

Site of injection and type of 5S rRNA	Amount of RNA in the nucleus relative to oocyte-type ^a	Storage 7S RNP (%) ^b	Preribosomal 5S RNP (%) ^b
Cytoplasmic injection			
Oocyte-type	1.0	43.9 ± 8.2 (5)	8.7 ± 5.8 (5)
Somatic-type	2.1 ± 0.4 (6)	8.0 ± 5.5 (5)	66.6 ± 18.5 (5)
Nuclear injection (RNA)			
Oocyte-type	1.0	20.3 (1)	8.7 (1)
Somatic-type	1.3 ± 0.1 (3)	15.1 ± 4.4 (2)	70.0 ± 1.3 (2)
Nuclear injection (DNA)			
Oocyte-type	1.0	9.4 ± 1.6 (4)	20.2 ± 2.5 (4)
Somatic-type	0.9 ± 0.2 (4)	6.1 ± 1.2 (2)	18.9 ± 0.1 (2)

^a Nuclear transport after approximately 20 hr was analyzed as described in Fig. 2. Data are expressed in arbitrary units as the mean ± the standard deviation relative to the amount of oocyte-type 5S rRNA in the nucleus. The number of experimental repetitions is indicated in parentheses.

^b 7S RNP and 5S RNP formation after 45 hr was analyzed by nondenaturing gel electrophoresis as described in Fig. 3. The percentage of bound RNA relative to free RNA was determined by densitometry.

of postvitellogenic oocytes, we analyzed the behavior of oocyte-type and somatic-type 5S rRNA after microinjection into previtellogenic to midvitellogenic oocytes (stages II, III, and IV). For this analysis the nucleus or cytoplasm could not be specifically targeted for microinjection, because the animal and vegetal hemispheres are undifferentiated in stage II and III oocytes, and the stage IV oocyte nucleus may not be displaced toward the animal pole (Dumont, 1972). As in fully grown oocytes, in stages II, III, and IV, microinjected oocyte-type 5S rRNA associated to a greater extent with TFI_{II}A than with L5, while somatic-type predominantly associated with L5 (Fig. 4). In these immature oocytes, somatic-type 5S rRNA that was associated with TFI_{II}A formed a complex of slightly altered mobility when compared with 7S RNPs containing oocyte-type 5S rRNA. Interestingly, oocyte-type was predominantly associated with the putative p43-5S rRNA complex, i.e., the higher molecular weight complex migrating more slowly than the 7S RNP and 5S RNP bands (cf. Fig. 3B and Fig. 4). Approximately 40% of the labeled RNA was found in this complex in stage II oocytes, decreasing to 15% in stage IV. In summary, these results confirm that throughout oogenesis microinjected oocyte-type 5S rRNA predominantly forms storage RNPs, whereas somatic-type 5S rRNA predominantly forms preribosomal 5S RNPs.

Somatic-Type 5S rRNA Is Assembled into Ribosomes

Having determined that somatic-type 5S rRNA is assembled into preribosomal 5S RNPs, we next addressed the question of whether microinjected somatic-type 5S rRNA can be assembled into oocyte ribosomes. 60S ribosomal subunits are not resolvable by the conditions of

electrophoresis described above, so ribosome assembly in stage II to VI oocytes was analyzed by immunoprecipitation with anti-60S ribosomal subunit antibodies. Densitometric analysis of the amount of somatic-type 5S rRNA immunoprecipitated as 60S ribosomal subunits, relative to oocyte-type 5S rRNA, is summarized in Table 2. Throughout oogenesis, differential assembly of the two types of microinjected RNA into ribosomes was observed. In previtellogenic, vitellogenic, and postvitellogenic oocytes a greater amount of somatic-type 5S rRNA was assembled into 60S ribosomal subunits when compared with oocyte-type 5S rRNA.

Approximately three times more somatic-type than oocyte-type 5S rRNA was found in 60S ribosomal subunits in stage II and III oocytes, and approximately four times more was found in stage IV oocytes (Table 2). In stage V-VI oocytes, the distribution of 60S ribosomal subunits between the nucleus and cytoplasm was analyzed by manual dissection of nuclear and cytoplasmic fractions, followed by immunoprecipitation. Figure 5 shows the nucleocytoplasmic distribution of oocyte-type and somatic-type 5S rRNA in 60S ribosomal subunits after introduction of RNA into either the cytoplasm or the nucleus. After cytoplasmic microinjection, approximately seven times more somatic-type 5S rRNA than oocyte-type 5S rRNA was found assembled into nascent 60S subunits (Fig. 5; Table 2). Even more striking was the difference in ribosome assembly after nuclear microinjection of RNA in excess of other ribosomal components. Nearly 30 times more somatic-type than oocyte-type 5S rRNA was found assembled into nascent 60S ribosomal subunits (Fig. 5; Table 2). 60S ribosomal subunits containing either oocyte-type or somatic-type 5S rRNA were primarily immunoprecipitated from the

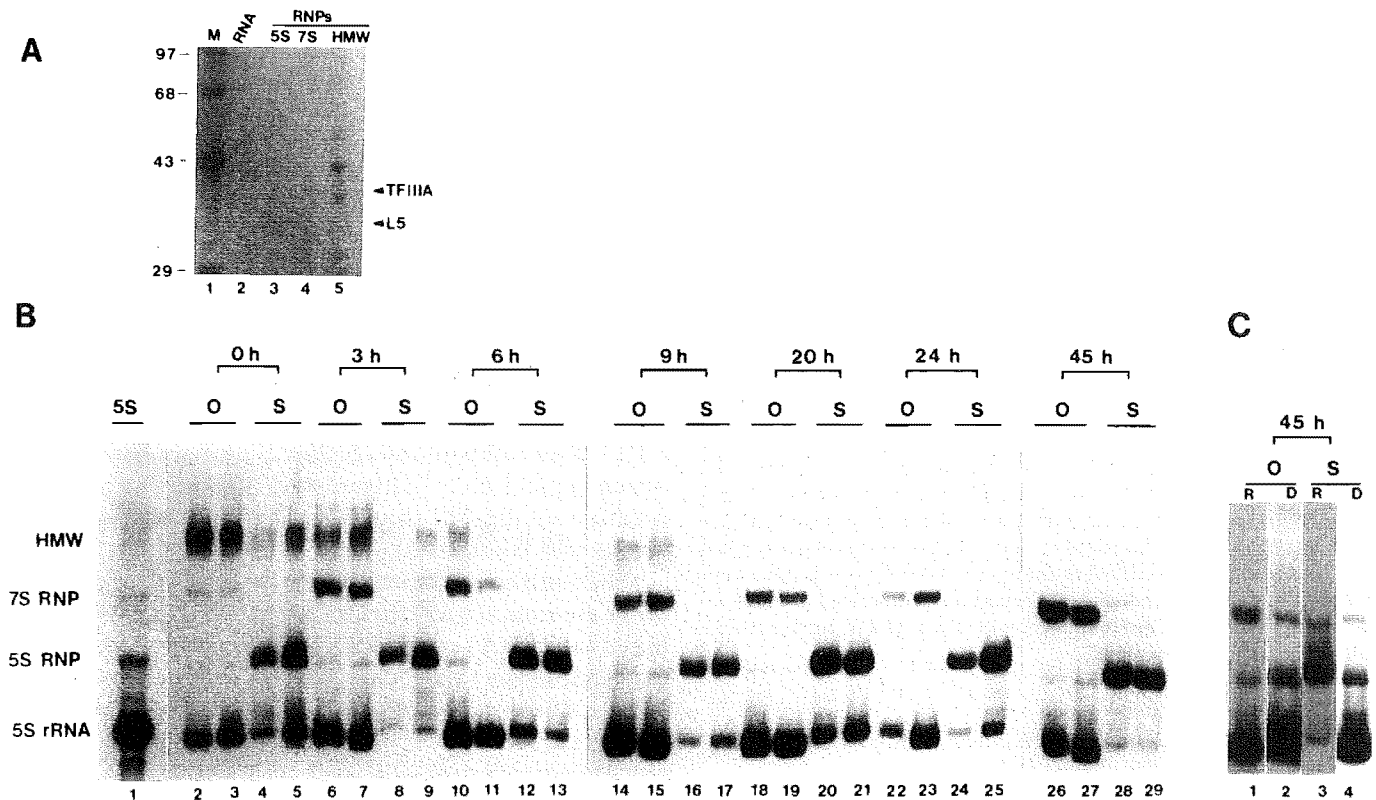


FIG. 3. Differential assembly of oocyte-type and somatic-type 5S rRNA into 7S RNPs and 5S RNPs. (A) Analysis of the components from 5S rRNA-containing RNPs. A 12% SDS-polyacrylamide gel stained with Coomassie blue shows the proteins present in bands excised from a gel similar to the one shown in B. Lanes: M, protein molecular mass markers (in kilodaltons); RNA, components of the unbound 5S rRNA band electroeluted directly into the protein gel; 5S, components of the 5S RNP band; 7S, components of the 7S RNP band; HMW, components of the higher molecular weight RNP band which may represent a p43-5S rRNA complex (see text). The ribosomal protein L5 band at 34 kDa and the TFIIA band at 38.5 kDa are shown by arrowheads. When a replicate gel was silver stained, an additional band was revealed in each lane, corresponding to 5S rRNA (data not shown). (B) ³²P-labeled oocyte-type or somatic-type 5S rRNA was injected into the oocyte cytoplasm. At the indicated times, cleared homogenates of three oocytes were subjected to nondenaturing gel electrophoresis. Duplicate samples were analyzed. The location of 7S RNPs was determined by running an additional lane of unlabeled, purified 7S RNPs; this lane was stained with ethidium bromide and visualized by uv illumination (not shown). 5S, 5S RNPs isolated from *Xenopus* ovary ribosomes, labeled by an *in vitro* exchange reaction with ³²P-5S rRNA. O, oocyte-type 5S rRNA. S, somatic-type 5S rRNA. HMW, higher molecular weight complex (see text). (C) Either labeled 5S rRNA (R) or [α-³²P]GTP and cloned 5S rRNA genes (D) was microinjected into the oocyte nucleus. The RNP distribution was analyzed after 45 hr incubation as described in B.

nucleus, in which subunit assembly occurs (Fig. 5). Longer exposures of the autoradiograms more clearly show the cytoplasmic pool of 60S ribosomal subunits (data not shown). In contrast, comparable amounts of oocyte-type and somatic-type 5S rRNA transcribed from microinjected gene templates were found in 60S ribosomal subunits (Fig. 5; Table 2), suggesting that when gradually synthesized *in situ* both types of RNA are rapidly and directly recruited into ribosomes, thus bypassing an excursion into the cytoplasm for storage prior to ribosome assembly.

DISCUSSION

5S rRNA Binding to TFIIA or L5: Specialization for Storage Versus Mobilization

In this study, we described experiments designed to test whether somatic-type 5S rRNA is capable of follow-

ing the individual steps in the pathway taken by oocyte-type 5S rRNA during oogenesis. We have shown that somatic-type 5S rRNA is indeed capable of RNP formation and nuclear transport; however, there are striking differences between oocyte-type and somatic-type 5S rRNA when the RNAs are introduced into the milieu of the oocyte cytoplasm or nucleus. We showed that microinjected oocyte-type and somatic-type 5S rRNA bind differentially to TFIIA and L5 and that these differences in protein binding are correlated with the behavior of the two types of RNA within the oocyte. In previtellogenic, vitellogenic, and postvitellogenic oocytes, microinjected oocyte-type 5S rRNA is complexed in storage RNPs, whereas somatic-type 5S rRNA in association with L5 is rapidly mobilized and targeted to the nucleus for ribosome assembly. These results suggest that the six nucleotide substitutions of oocyte-type 5S

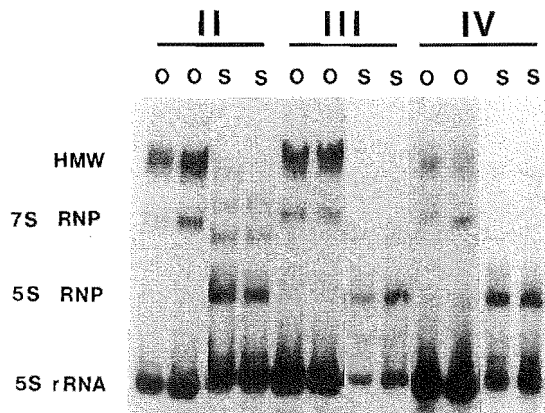


FIG. 4. Differential RNP distribution of oocyte-type and somatic-type 5S rRNA in previtellogenic and midvitellogenic oocytes. Labeled oocyte-type or somatic-type 5S rRNA was injected into stage II, III, or IV oocytes. After 24 hr incubation, cleared homogenates of five oocytes were electrophoresed on a nondenaturing gel. Duplicate samples were analyzed for 5S RNP and 7S RNP formation as described in Fig. 3. O, oocyte-type 5S rRNA. S, somatic-type 5S rRNA. HMW, higher molecular weight complex (see text).

rRNA are indeed advantageous for the unusual storage pathway taken during oogenesis.

Ribosomal protein L5 and 5S rRNA form a stable complex prior to assembly of ribosomal subunits in *Xenopus* oocytes; thus 5S rRNA must be exchanged from binding with TFIIA to binding with L5 prior to localization within the nucleolus and 60S ribosomal subunit assembly (Allison *et al.*, 1991). Since oocyte-type 5S rRNA rapidly forms storage 7S RNPs after microinjection into the cytoplasm, an additional step is required along the pathway to ribosome assembly. During stages I to III of oogenesis, TFIIA reaches its highest concentration, then decreases 10- to 20-fold during the later stages of oogenesis (Stages IV to VI) (Dixon and Ford, 1982; Ginsberg *et al.*, 1984). Thus, the majority of the interactions of oocyte-type 5S rRNA with TFIIA after cytoplasmic microinjection into stage V–VI oocytes probably reflect exchange of the labeled 5S rRNA for the endogenous RNA in pre-existing 7S RNPs (Andersen and Delihis, 1986; Allison *et al.*, 1991).

In contrast to the behavior of oocyte-type 5S rRNA, microinjected somatic-type complexes with L5 and is readily available for nuclear import and ribosome assembly. This finding that microinjected somatic-type 5S rRNA predominantly associates with L5 rather than with TFIIA was surprising, given that somatic-type binds to TFIIA with approximately 1.7 times greater affinity than oocyte-type 5S rRNA *in vitro* (Romaniuk *et al.*, 1987). However, it has been shown that somatic-type 5S rRNA is incorporated less efficiently than oocyte-type into new 7S RNPs in ovary homogenates (Denis and le Maire, 1983). Further, somatic-type 5S rRNA also binds with greater affinity to L5 than oocyte-type *in vi-*

tro (Q. You, W. Q. Zang, and P. J. Romaniuk, in preparation). Our observations suggest that somatic-type 5S rRNA also binds to L5 with high affinity *in vivo*. Although L5 and TFIIA recognize a common binding site on 5S rRNA, these two proteins share no sequence similarity and are subject to distinct developmental regulation (Wormington, 1989). The synthesis of L5 becomes maximal in stage III oocytes, coordinated with expression of 18S–5.8S–28S rRNA and ribosome assembly (Wormington, 1989). Thus, the stages of oocytes used in our experiments have a relatively abundant pool of L5 protein, some of which may be free to associate with exogenous 5S rRNA. Alternatively, microinjected 5S rRNA may compete for L5 that is already bound to endogenous 5S rRNA (Allison *et al.*, 1991) by an exchange reaction similar to that carried out *in vitro* (Fig. 3B; Huber and Wool, 1986).

When introduced into the oocyte nucleus in excess to other ribosomal constituents, oocyte-type preferentially binds to TFIIA to form 7S RNPs, some of which may be exported to the cytoplasm. TFIIA plays a role in mediating nuclear export of 5S rRNA (Guddat *et al.*, 1990); however, once in the cytoplasm, 7S RNPs are retained as storage particles (Mattaj *et al.*, 1983; Allison *et al.*, 1991, 1993). Somatic-type 5S rRNA, on the other hand, preferentially binds to L5 and thus is more rapidly assembled into ribosomes as the other ribosomal components be-

TABLE 2
ASSEMBLY OF SOMATIC-TYPE 5S rRNA INTO 60S RIBOSOMAL SUBUNITS DURING OOGENESIS

Stage of oogenesis	60S subunit assembly relative to oocyte-type 5S rRNA ^a
Stage II	2.8 ± 0.8 (2)
Stage III	2.9 ± 0.1 (2)
Stage IV	4.3 ± 0.0 (2)
Stage V–VI	
Cytoplasmic injection	7.0 ± 3.3 (4)
Nuclear injection (RNA)	29.8 ± 4.7 (2)
Nuclear injection (DNA)	1.2 ± 0.6 (3)

^a Assembly of somatic-type 5S rRNA into 60S ribosomal subunits was analyzed by immunoprecipitation as described in Fig. 5, except assays were carried out on cleared homogenates of whole stage II to IV oocytes. Data from densitometric analysis are expressed in arbitrary units as the mean ± the standard deviation relative to oocyte-type 5S rRNA, which was assigned a value of 1.0, after adjusting for any minor differences in the total amount of ³²P-labeled RNA present in oocyte homogenates. The total amount of radioactivity expressed as the average counts per minute (cpm), recovered from RNA extracted from immunoprecipitates and immunosupernatants, was as follows. Stages II to IV: oocyte-type, 2260 cpm; somatic-type, 1620. Stage V–VI cytoplasmic injection: oocyte-type, 15,510 cpm; somatic-type, 20,350 cpm. Nuclear injection (RNA): oocyte-type, 10,800 cpm; somatic-type, 9010 cpm. Nuclear injection (DNA): oocyte-type, 1260; somatic-type, 1750 cpm. The number of experimental repetitions is indicated in parentheses.

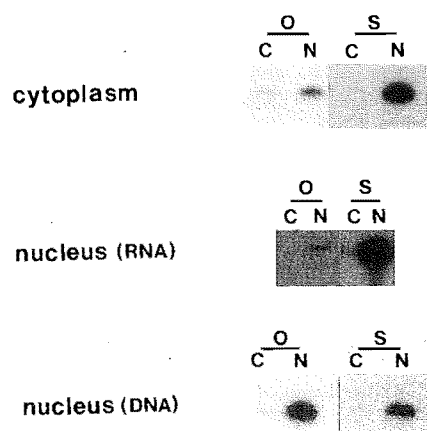


FIG. 5. Assembly of somatic-type 5S rRNA into 60S ribosomal subunits. ^{32}P -labeled oocyte-type or somatic-type 5S rRNA was microinjected into the oocyte cytoplasm or nucleus, or gene templates (DNA) were injected with $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ into the nucleus. After 45 hr, oocytes were manually fractionated into cytoplasmic (C) and nuclear (N) compartments. Cleared homogenates from each compartment were incubated with anti-60S ribosomal subunit antibodies bound to protein A-Sepharose in immunoprecipitation assays. Labeled RNAs were recovered and separated by denaturing polyacrylamide gel electrophoresis. O, oocyte-type 5S rRNA. S, somatic-type 5S rRNA.

come available with time. In contrast, when gradually synthesized *in situ*, both types of RNA are primarily associated with L5, and comparable amounts are found in 60S ribosomal subunits. Thus, nascent 5S rRNA can apparently bypass an excursion into the cytoplasm prior to ribosome assembly and participate in ribosome biogenesis via the typical somatic cell pathway. This result concurs with an earlier study by Mairy and Denis (1972) showing that newly synthesized endogenous 5S rRNA is taken up by ribosomes in vitellogenic oocytes somewhat more rapidly than stored 5S rRNA. No distinction was made between oocyte-type and somatic-type 5S rRNA by these authors, since their study was carried out prior to the identification of the sequence heterogeneity of 5S rRNA.

The recruitment of somatic-type 5S rRNA into stage II to VI oocyte ribosomes shown here corroborates earlier reports that provided indirect evidence for incorporation of somatic cell-type 5S rRNA into oocyte ribosomes. Guddat *et al.* (1990) found partial recruitment in fully grown oocytes of newly transcribed *Xenopus* somatic-type 5S rRNA into RNPs that sediment at about 80S on glycerol gradients, and HeLa cell (human) 5S rRNA was shown to be correctly targeted to the nucleus and to the nucleoli in fully grown *Xenopus* oocytes indicating incorporation into preribosomal particles (DeRobertis *et al.*, 1982). Five of the *Xenopus* 5S rRNA somatic-specific substitutions are conserved in human 5S rRNA (Garrett *et al.*, 1991); thus these observations are not unexpected.

Our study does not address the question of differential

stability over longer time periods or of functional activity. Ribosomes that contain somatic-type 5S rRNA may be suitable for short-term use in protein synthesis in the developing oocyte. Maternal ribosomes are stored many months in the oocyte and then after fertilization function in the synthesis of embryonic proteins until the swimming tadpole stage (Miller, 1974). Somatic-type 5S rRNA has not been detected in this pool of stored ribosomes, suggesting that perhaps the conformation of somatic-type 5S rRNA is different enough from that of oocyte-type 5S rRNA to confer lower metabolic stability to ribosomes which take up somatic-type 5S rRNA (Denis and Wegnez, 1977). Although somatic-type and oocyte-type 5S rRNAs adopt very similar conformations *in vitro* (Westhof *et al.*, 1989), helices III and IV in somatic-type are less stable than the same structures in oocyte-type 5S rRNA (Romaniuk *et al.*, 1988).

The Pathway of 5S rRNA to the Ribosome

Is the pathway followed by 5S rRNA for assembly into ribosomes in oocytes peculiar to these specialized cells or does some of the 5S rRNA in somatic cells follow a similar pathway? As in oocytes, L5 and 5S rRNA form a stable complex prior to assembly of ribosomal subunits in somatic cells (Steitz *et al.*, 1988; Wormington, 1989). TFIIA has the distinctive ability to bind specifically to both 5S rRNA genes and 5S rRNA of oocyte-type and somatic-type (Engelke *et al.*, 1980; Honda and Roeder, 1980; Pelham and Brown, 1980; Kim *et al.*, 1990; Pfaff *et al.*, 1991; Theunissen *et al.*, 1992; Bogenhagen, 1993); however, it is not clear whether storage RNPs, analogous to those found in oocytes, form in somatic cells. In mammalian cells, excess 5S rRNA is thought to be targeted to the cytoplasm for degradation bound to a TFIIA-like protein (Lagaye *et al.*, 1988). Another recent report describes a pool of cytoplasmic 5S rRNA in rat liver present in a large complex containing aminoacyl-tRNA synthetases (Ogata *et al.*, 1993). The significance of these complexes *in vivo* remains unknown. It seems unlikely that cytoplasmic 5S rRNA in somatic cells would return to the nucleus and be incorporated into nascent ribosomes, given that assembly of ribosomal components in somatic cells appears to require concurrent synthesis of all components (Craig and Perry, 1971; Miller, 1974).

The somatic-type 5S rRNA sequence appears to be more closely related to ancestral 5S rRNA than oocyte-type, and it is thought that the mutations which have accumulated in oocyte-type 5S rRNA are unlikely to be neutral (Ford and Southern, 1973). We conclude that the oocyte-specific nucleotide substitutions are indeed advantageous and result in distinct functional differences that specialize oocyte-type 5S rRNA for following a pathway of ribosome biogenesis that involves additional protein associations, bidirectional nuclear transport, and long-term storage in the cytoplasm.

We are grateful to Renny Bishop for maintaining the *Xenopus* colony. We thank Paul Romaniuk for providing the plasmids pXlo-wt and pXls-wt, Aimée Bakken for the plasmids pXlo8 and pJH17, and Marc le Maire for the anti-60S antiserum. Thanks are also due to Martin Zillman for sharing a highly efficient T7 RNA polymerase transcription protocol. Finally, we thank Paul Romaniuk, Aimée Bakken, Marc le Maire, Stéphane Deschamps, Sylvia Nagl, and Kirstie Murdoch for their helpful criticism of the manuscript. This research was supported in part by grants from the Lottery Grants Board and Health Research Council of New Zealand to L.A.A. M.N. is the recipient of postgraduate scholarships from the Ministry of Research, Science, and Technology and the University of Canterbury. 5S RNP characterization and exchange reactions were carried out by L.N. in partial fulfillment of the requirements for a Bachelor of Science (Honors) degree.

REFERENCES

- Allison, L. A., North, M. T., Murdoch, K. J., Romaniuk, P. J., Deschamps, S., and le Maire, M. (1993). Structural requirements of 5S rRNA for nuclear transport, 7S ribonucleoprotein particle assembly, and 60S ribosomal subunit assembly in *Xenopus* oocytes. *Mol. Cell. Biol.* **13**, 6819-6831.
- Allison, L. A., Romaniuk, P. J., and Bakken, A. H. (1991). RNA-protein interactions of stored 5S RNA with TFIIA and ribosomal protein L5 during *Xenopus* oogenesis. *Dev. Biol.* **144**, 129-144.
- Andersen, J., and Delias, N. (1986). Characterization of RNA-protein interactions in 7S ribonucleoprotein particles from *Xenopus laevis* oocytes. *J. Biol. Chem.* **261**, 2912-2917.
- Birkenmeier, E. H., Brown, D. D., and Jordon, E. (1978). A nuclear extract of *Xenopus laevis* oocytes that accurately transcribes 5S RNA genes. *Cell* **15**, 1077-1086.
- Bogenhagen, D. F. (1993). Proteolytic footprinting of transcription factor TFIIA reveals different tightly binding sites for 5S RNA and 5S DNA. *Mol. Cell. Biol.* **13**, 5149-5158.
- Craig, N., and Perry, R. P. (1971). Persistent cytoplasmic synthesis of ribosomal proteins during the selective inhibition of ribosomal RNA synthesis. *Nature New Biol.* **229**, 75-80.
- Denis, H., and le Maire, M. (1983). Thesaurisomes, a novel kind of nucleoprotein particle. In "Subcellular Biochemistry" (D. B. Roodyn, Ed.), pp. 263-267. Plenum, New York.
- Denis, H., and Wegnez, M. (1977). Biochemical research on oogenesis. Oocytes of *Xenopus laevis* synthesize but do not accumulate 5S RNA of somatic type. *Dev. Biol.* **58**, 212-217.
- DeRobertis, E. M., Lienhard, S., and Parisot, R. F. (1982). Intracellular transport of microinjected 5S and small nuclear RNAs. *Nature* **295**, 572-577.
- Dixon, L. K., and Ford, P. J. (1982). Regulation of protein synthesis and accumulation during oogenesis in *Xenopus laevis*. *Dev. Biol.* **93**, 478-497.
- Dumont, J. N. (1972). Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocyte development in laboratory maintained animals. *J. Morphol.* **136**, 153-180.
- Engelke, D. R., Ng, S.-Y., Shastry, B. S., and Roeder, R. G. (1980). Specific interaction of a purified transcription factor with an internal control region of 5S RNA genes. *Cell* **19**, 717-728.
- Ford, P. J., and Southern, E. M. (1973). Different sequences for 5S RNA in kidney cells and ovaries of *Xenopus laevis*. *Nature New Biol.* **241**, 7-12.
- Garrett, R. A., Douthwaite, S., and Noller, H. F. (1981). Structure and role of 5S RNA-protein complexes in protein biosynthesis. *Trends Biochem. Sci.* **6**, 137-139.
- Ginsberg, A. M., King, B. O., and Roeder, R. G. (1984). *Xenopus* 5S gene transcription factor, TFIIA: Characterization of a cDNA clone and measurement of RNA levels throughout development. *Cell* **39**, 479-489.
- Gottlieb, E., and Steitz, J. A. (1989). Function of the mammalian La protein: Evidence for its action in transcription termination by RNA polymerase III. *EMBO J.* **8**, 851-861.
- Guddat, U., Bakken, A. H., and Pieler, T. (1990). Protein-mediated nuclear export of RNA: 5S rRNA containing small RNPs in *Xenopus* oocytes. *Cell* **60**, 619-628.
- Gurdon, J. B., and Melton, D. A. (1981). Gene transfer in amphibian eggs and oocytes. *Annu. Rev. Genet.* **15**, 189-218.
- Hadjiolov, A. A. (1985). "The Nucleolus and Ribosome Biogenesis." Springer-Verlag, New York.
- Honda, B. M., and Roeder, R. G. (1980). Association of a 5S gene transcription factor with 5S RNA and altered levels of the factor during cell differentiation. *Cell* **22**, 119-126.
- Huber, P. W., and Wool, I. G. (1986). Use of the cytotoxic nuclease α -sarcin to identify the binding site on eukaryotic 5S ribosomal ribonucleic acid for the ribosomal protein L5. *J. Biol. Chem.* **261**, 3002-3005.
- Jarmolowski, A., Boelens, W. B., Izaurralde, E., and Mattaj, I. W. (1994). Nuclear export of different classes of RNA is mediated by specific factors. *J. Cell Biol.* **124**, 627-635.
- Keller, H. J., You, Q., Romaniuk, P. J., and Gottesfeld, J. M. (1990). Additional intragenic promoter elements of the *Xenopus* 5S RNA genes upstream from the TFIIA-binding site. *Mol. Cell. Biol.* **10**, 5166-5176.
- Kim, S. H., Darby, M. K., Joho, K. E., and Brown, D. D. (1990). The characterization of the TFIIA synthesized in somatic cells of *Xenopus laevis*. *Genes Dev.* **4**, 1602-1610.
- Lagaye, S., Barque, J.-P., le Maire, M., Denis, H., and Larsen, C.-J. (1988). Characterization by human antibodies of two HeLa cell proteins which are related to *Xenopus laevis* transcription factor TFIIA. *Nucleic Acids Res.* **16**, 2473-2487.
- Mairy, M., and Denis, H. (1971). Recherches biochimiques sur l'oogenèse. 1. Synthèse et accumulation du RNA pendant l'oogenèse du crapaud sud-africain *Xenopus laevis*. *Dev. Biol.* **24**, 143-165.
- Mairy, M., and Denis, H. (1972). Recherches biochimiques sur l'oogenèse. 2. Assemblage des ribosomes pendant le grand accroissement des oocytes de *Xenopus laevis*. *Eur. J. Biochem.* **25**, 535-543.
- Mattaj, I. W., Lienhard, S., Zeller, R., and DeRobertis, E. M. (1983). Nuclear exclusion of transcription factor IIA and the 42S particle transfer RNA-binding protein in *Xenopus* oocytes: A possible mechanism for gene control. *J. Cell Biol.* **97**, 1261-1265.
- McConkey, G. A., and Bogenhagen, D. F. (1988). TFIIA binds with equal affinity to somatic and major oocyte 5S RNA genes. *Genes Dev.* **2**, 205-214.
- Miller, L. (1974). Metabolism of 5S RNA in the absence of ribosome production. *Cell* **3**, 275-281.
- Ogata, K., Kurahashi, A., Nishiyama, C., and Terao, K. (1993). Metabolic characteristics of rat liver cytosolic 5S RNA. *Eur. J. Biochem.* **213**, 1277-1282.
- Pelham, H. R. B., and Brown, D. D. (1980). A specific transcription factor that can bind either the 5S RNA gene or 5S RNA. *Proc. Natl. Acad. Sci. USA* **77**, 4170-4174.
- Pfaff, S. L., Hall, R. K., Hart, G. C., and Taylor, W. L. (1991). Regulation of the *Xenopus laevis* transcription factor IIA gene during oogenesis and early embryogenesis: Negative elements repress the O-TFIIA promoter in embryonic cells. *Dev. Biol.* **145**, 241-254.
- Picard, B., and Wegnez, M. (1979). Isolation of a 7S particle from *Xenopus laevis* oocytes: A 5S RNA-protein complex. *Proc. Natl. Acad. Sci. USA* **76**, 241-245.
- Picard, B., le Maire, M., Wegnez, M., and Denis, H. (1980). Biochemical research on oogenesis. Composition of the 42-S storage particles of *Xenopus laevis* oocytes. *Eur. J. Biochem.* **109**, 359-368.
- Romaniuk, P. J., Leal de Stevenson, I., Ehresmann, C., Romby, P., and Ehresmann, B. (1988). A comparison of the solution structures and conformational properties of the somatic and oocyte 5S rRNAs of *Xenopus laevis*. *Nucleic Acids Res.* **16**, 2295-2312.
- Romaniuk, P. J., Leal de Stevenson, I., and Wong, H.-H. A. (1987). De-

- fining the binding site of *Xenopus* transcription factor IIIA on 5S RNA using truncated and chimeric 5S RNA molecules. *Nucleic Acids Res.* **15**, 2737-2755.
- Sands, M. S., and Bogenhagen, D. F. (1991). Two zinc finger proteins from *Xenopus laevis* bind the same region of 5S RNA but with different nuclease protection patterns. *Nucleic Acids Res.* **19**, 1797-1803.
- Steitz, J. A., Berg, C., Hendrick, J. P., La Branche-Chabot, H., Metspalu, A., Rinke, J., and Yario, T. (1988). A 5S rRNA/L5 complex is a precursor to ribosome assembly in mammalian cells. *J. Cell Biol.* **106**, 545-556.
- Theunissen, O., Rudt, F., Guddat, U., Mentzel, H., and Pieler, T. (1992). RNA and DNA binding zinc fingers in *Xenopus* TFIIA. *Cell* **71**, 679-690.
- Viel, A., Armand, M.-J., Callen, J.-C., Gomez de Gracia, A., Denis, H., and le Maire, M. (1990). Elongation factor 1 α (EF-1 α) is concentrated in the Balbiani body and accumulates coordinately with the ribosomes during oogenesis of *Xenopus laevis*. *Dev. Biol.* **141**, 270-278.
- Wegnez, M., Monier, R., and Denis, H. (1972). Sequence heterogeneity of 5S RNA in *Xenopus laevis*. *FEBS Lett.* **25**, 13-20.
- Westhof, E., Romby, P., Romaniuk, P. J., Ebel, J.-P., Ehresmann, C., and Ehresmann, B. (1989). Computer modeling from solution data of spinach chloroplast and of *Xenopus laevis* somatic and oocyte 5S rRNAs. *J. Mol. Biol.* **207**, 417-431.
- Wolffé, A. P., and Brown, D. D. (1988). Developmental regulation of two 5S ribosomal RNA genes. *Science* **241**, 1626-1632.
- Wormington, W. M. (1989). Developmental expression and 5S rRNA-binding activity of *Xenopus laevis* ribosomal protein L5. *Mol. Cell Biol.* **9**, 5281-5288.
- Wormington, W. M., and Brown, D. D. (1983). Onset of 5S RNA gene regulation during *Xenopus* embryogenesis. *Dev. Biol.* **99**, 248-257.
- Wormington, W. M., Bogenhagen, D. F., Jordan, E., and Brown, D. D. (1981). A quantitative assay for *Xenopus* 5S RNA gene transcription *in vivo*. *Cell* **24**, 809-817.
- Xie, W. Q., and Rothblum, L. I. (1991). Rapid, small-scale RNA isolation from tissue culture cells. *BioTechniques* **11**, 324-327.